Reply Brief Transmittal

In the United States Patent and Trademark Office
Board of Patent Appeals and Interferences (37 CFR 1.191)

In re application of: James P. Elia

Docket No.: 1000-10-CO1

Serial No. 09/836,750

Group No.: 1646

Filed: April 17, 2001

Examiner: Elizabeth C. Kemmerer, Ph.D.

For: METHOD FOR GROWING MUSCLE IN A HUMAN HEART

MAIL STOP APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Appellant hereby submits a **Reply Brief** in the above-identified application.

The item(s) checked below are appropriate:

1. **Status of Applicant/Appellant**
This application is on behalf of
[X] a small entity

2. **Extension of Term**

[X] No Extension requested.

Date: March 14, 2008

Reg. No.: 26,611

Gerald K. White
Signature of attorney

Gerald K. White
Type or print name of attorney

CERTIFICATE OF MAILING

I hereby certify that the attached APPELLANT'S REPLY BRIEF was deposited as First Class Mail, in an envelope addressed to Mail Stop APPEAL BRIEF-PATENTS, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 this 14th day of March, 2008.

Dated: March 14, 2008

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REPLY BRIEF

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STATUS OF CLAIMS AND CLAIMS UNDER APPEAL

Claims 1-5 were cancelled in the Preliminary Amendment filed April 17, 2001.

Claims 204, 205, and 237 were cancelled in the Amendment filed February 17, 2004.

Claims 254-256 were cancelled in the Amendment filed November 21, 2005.

Claims 6-235 and 240-242 stand withdrawn, by the Examiner, from consideration as being directed to a non-elected invention. Claims 204 and 205 were cancelled by Appellant and thus were incorrectly identified by the Examiner as being withdrawn.

Claims 245, 246, 248, 249, 252, 264-267, 272-279, 286, and 287 were cancelled in the Amendment filed October 15, 2007.

In view of the above-identified withdrawal and cancellation of claims, the correctness of the Examiner's Final Rejection of claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 under 35 U.S.C. §112, first paragraph, for lack of enablement constitutes the sole issue on appeal.

STATUS OF AMENDMENTS

The sole amendment made or entered subsequent to the Final Rejection of September 22, 2006 is the Amendment filed October 15, 2007 canceling claims 245, 246, 248, 249, 252, 264-267, 272-279, 286, and 287. In the Examiner's Answer dated January 24, 2008 (hereinafter "the Answer"), the Examiner stated that the Amendment had been entered.

GROUNDS OF REJECTION FOR REVIEW ON APPEAL

Appealed claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 stand finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

ARGUMENT

Rejection of Claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 Under 35 U.S.C. §112, First Paragraph

The Answer contains inaccuracies and raises a number of new issues which are addressed below in the instant Reply Brief. The inaccuracies and new issues are addressed in chronological order for the convenience of the reader.

At page 8, paragraph 4 of the Answer, the Examiner raises a new issue regarding timing of treatment by citing Murry et al. (hereinafter "Murry"). Apparently, the Examiner relies upon Murry, for the first time in this record, to bolster the prior allegation that timing of treatment required a great amount of experiment. Note that the paragraph bridging pages 21 and 22 of the Answer does not rebut Appellant's prior assertion that Strauer does not support the Examiner's position regarding timing of treatment. For the Examiner to now disregard the three Strauer et al. publications (hereinafter "Strauer 2002, 2003, and 2005" respectively and all of record) that show that both acute and chronic conditions are treatable and further cite Murry to support her position is not understood by Appellant. Murry discloses transplanting skeletal muscle in the heart of a murine model, while Strauer 2002, 2003, and 2005 disclosed the formation of new arteries and cardiac muscle in the heart of human patients. Surely, evidence from Strauer 2002, 2003, and 2005 is clearly more probative of the timeliness issue because such evidence tracks the claimed subject matter.

The Examiner at page 14, lines 1 and 2 and at page 33, lines 12 and 13, concludes that growth of new arteries to repair dead/damaged heart tissue by cell therapy has not

been achieved by workers in the art. Appellant agrees that the state of the art at the time the instant application was filed does not disclose the growth of new arteries. However, there can be no doubt that post-filing publications, including Orlic et al., cited previously by the Examiner (hereinafter "Orlic"); Strauer 2002; and Dohmann et al. (copy furnished by the Examiner in the Answer and hereinafter "Dohmann") confirm Appellant's disclosed and claimed results, i.e., heart repair and formation of a new artery and new cardiac muscle.

Orlic describes injecting bone marrow stem cells into murine models having infarcted hearts and provides autopsy confirmation of the formation of arteries and arterioles (small arteries). Note that Orlic, as well as Dohmann, used the term "smooth muscle actin" to positively identify the formation of a new artery in the respective autopsies. Orlic used enhanced green fluorescent protein to identify the formation of endothelial cells. Unlike capillaries, which are composed of endothelial cells, arteries and arterioles additionally require smooth muscle cells. In this regard, please see Orlic at page 702, left column, top of page, where it is stated:

> This allowed us to identify each cardiac cell type and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a-c; see also Supplementary Information). The percentages of <u>new</u> (*emphasis added by Appellant*) myocytes, endothelial cells, and smooth muscle cells expressing EGFP was 53 +/- 9% (n=7), 44 +/- 6%) (n=7) and 49 +/- 7% (n=7), respectively.

From the foregoing, one skilled in the medical art would have no difficulty in recognizing the Orlic work resulted in growth of new arteries, as well as the growth of new cardiac muscle.

Strauer 2002 used the term "neovascularization" (page 1913, abstract) to describe the results of the administration of mononuclear bone marrow stem cells to a heart of a human patient to achieve repair. Obviously, neovascularization includes new artery formation, not merely capillary formation, in view of the impressive improvement (page 1917, top of page) in heart function described therein. Note further at page 1916, right column, paragraph 1 to where it is disclosed:

> ... in several animal infarction models it has been shown that: (1) bone marrow hemangioblasts contribute to the formation of new vessels (*emphasis added by Appellant*); (2) bone marrow hematopoietic stem cells differentiate into cardio myocytes, endothelium, and smooth muscle cells [ref 8-13...

Note that ref 11 of Strauer 2002 in the above quotation is the Orlic publication. Being that Strauer 2002 and Orlic administered bone marrow stem cells to achieve heart repair, one skilled in the medical art would readily recognize that Strauer 2002 also formed new arteries and muscle.

Contrary to the Examiner's inaccurate, new allegation, Dohmann provides evidence that earlier trials involving implantation of bone marrow stem cells resulted in new artery and new muscle formation and heart repair via autopsy confirmation on a trial patient. Such trials involved injection (page 2, abstract) of bone marrow stem cells into the heart of a human patient to repair the heart. Please note that Figures 3C, D, and E on page 9 of Dohmann indicate that the smooth muscle walls comprise many cell thicknesses of smooth muscle and that smooth muscle actin was identified in the autopsy. As is well known in the medical art, capillaries comprise tubes of one cell thickness

devoid of smooth muscle cells. An artery must include at least endothelial and smooth muscle cells.

At page 8, under "Immunocytochemistry Findings," lines 6-8, Dohmann states that:

> The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls (*emphasis added by Appellant*), which had a marked hypertrophy of smooth muscle cells (Figure 3 D).

Dohmann uses the word "new" to describe the formation of blood vessels. See page 11, paragraph 3, line 1 to the bottom of the page in this regard. Inasmuch as Dohmann, along with Orlic and Strauer 2002, describe obtaining endothelial cells and smooth muscle cells, there can be no doubt that the formed new blood vessels include newly formed arteries. Such disclosure is clearly contrary to the Examiner's inaccurate, new, and unsupported assertion that a new artery is not grown and the new assertion that Dohmann avoided stating that new arteries had been formed.

The Examiner at page 33 of the Answer stated for the first time that, "Dohmann et al. state that some of the cells had acquired cytoskeletal elements and contractile proteins; however...carefully avoid concluding that arteries had been formed in this peer-reviewed publication." Such statement is confusing and is not relevant to the issue of whether or not new arteries were grown. The main thrust of Dohmann was to support the efficacy of stem cell therapy by publishing that no adverse histological effects were attributed to stem cell injections.

Rather than rely upon the statement in the prior paragraph, the Examiner should have considered the CONCLUSIONS section at page 3 of Dohmann where it was

reported that an "active process of angiogenesis was present" and "regeneration of the cardiac vascular tree and muscle" was confirmed by human autopsy. Moreover, Dohmann's statements regarding smooth muscle on the vessels and Figure 3 of the publication illustrating histological cross-sections of arteries constitute further confirmation to one skilled in the art that new arteries and muscle were grown.

The fact that Appellant, Orlic, Strauer 2002, and Dohmann all administer bone marrow stem cells to achieve heart repair and that all three publications describe new artery formation would be readily understood by one skilled in the art as demonstrated above. Either the Examiner does not recognize that arteries necessarily contain endothelial cells and smooth muscle cells or is artfully relying upon a "*de novo*" artery claim construction to reach the conclusion that Orlic, Strauer 2002, and Dohmann do not form new arteries within the scope of the claims. Either approach constitutes error.

Appellant submits that the Examiner has inaccurately based the above statement upon a failure of post-filing date publications to "report the *de novo* growth of an artery as defined by Appellant, including Strauer et al 2002." As demonstrated below, the term *de novo* artery is a fabrication of the Examiner and was never used by Appellant or any of the publications of record to define formation of a new artery.

The Examiner inaccurately defines/limits Appellant's claimed invention as requiring a *de novo* artery rather than reading and comprehending the specification and claims. Such erroneous approach violates the tenants of Phillips v. AWH., Corp., 415 F. 3d 1303 (Fed. Cir. 2005) where claim terms are to be interpreted in the context of the specification. Instead, the Examiner coined the term *de novo* which remains undefined or authenticated by the Examiner in the field of the invention. The newly alleged

"translation" of *de novo* as "anew" at page 14 of the Answer has no nexus with the disclosed/claimed invention. Such purported, unsubstantiated "translation" does not provide evidence which supports the Examiner's determination that the claims require and are limited to *de novo* formation of arteries. The Examiner erred in conditioning the enablement determination upon such oblique claim construction.

Also, at pages 8 and 9, paragraph 5 of the Answer, the Examiner erroneously alleges for the first time that Appellant attempted to support enablement with "numerous post-filing date publications..." It is clear from the record that Appellant never relied upon any extraneous post-filing date publications to support enablement, including the Strauer 2002 publication. Rather, Appellant has consistently relied upon the specification of the instant patent application and the unchallenged fact that the materials and techniques needed to practice the invention were known in the art individually, but not in combination, to enable one skilled in the art to form a new artery. Declarants, Dr. Richard Heuser and Dr. Andrew E. Lorincz, likewise relied upon the specification as a basis for their respective opinions that one skilled in the medical art would be enabled to make and use the claimed invention.

Also at pages 8 and 9, paragraph 5 of the Answer, the Examiner, for the first time, addresses the subject matter of claims 270 and 271 as requiring "localized administration at the site of dead or damaged myocardium." The Examiner cites both pre- and post-dated publications as evidence that only intramuscular injection of cells directly into the myocardium could successfully treat a damaged heart but argues that none of these publications show the growth of *de novo* arteries. Appellant does not understand the

nexus between these publications and the issue of enablement in regard to the subject matter of claims 270 and 271.

At page 12 of the Answer, the Examiner raises a new issue that actual experiments (working examples/clinical trials) are required to enable a patent application, yet fails to specifically identify what protocol is missing from Appellant's specification that would prevent one skilled in the art from practicing the claimed subject matter. The Examiner's above-referenced new remarks involving "writing it down," absent evidence or sound reasoning, is insufficient to overcome the objective enablement provided by the specification. In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Apparently, the Examiner fails to appreciate that the act of "writing down" a "prophetic" example, which describes an embodiment based upon predicted results rather than work actually conducted, is sufficient to satisfy a constructive reduction to practice. See MPEP 2164.02 and cited case law that stands for the proposition "the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it." In re Chilowsky, 29 F. 2d 457, 461, 108 USPQ 321, 325 (CCPA 1956). The use of prophetic examples does not automatically render a specification non-enabled. The burden is on the Examiner, when challenging enablement, to show by clear and convincing evidence that the prophetic examples, when combined with the disclosure as a whole, do not make a case of enablement. It is clear on this record that the Examiner has not discharged such a burden. Cf. Amgen, Inc.v. Chugai Pharmaceutical Co., 927 F2d. 1200, 18 USPQ 2d 1016 (Fed. Cir.), cert. denied, 502 U.S. 856, (1991). Furthermore, the Examiner's requirement for experimentation appears to be in contrast with current Patent and Trademark Office

(hereinafter "PTO") practice. In rebuttal to this new issue, Appellant points to recently issued U. S. Patent No. 7,097,832 granted to Kornowski et al.[1] (attached hereto as Exhibit A). Said patent contains claims drawn to a cell therapy treatment of humans requiring the implantation of stem cells in the heart to grow collateral blood vessels based on a prophetic disclosure. Further, the Examiner's gratuitous reference to unclaimed inventions is irrelevant to the issue on appeal.

The Examiner at pages 14, 15, and 16 of the Answer, for the first time, inaccurately cites descriptive language from three portions of the specification which purportedly establish that Appellant's invention requires the formation of *de novo* cardiac muscle and *de* novo arteries. Appellant submits that a cursory reading of such descriptive language establishes the inaccuracy of the Examiner's position.

First, the Examiner failed to specifically point out wherein the specification uses the term "*de novo,*" and for good reason. The Examiner has filed to point to any specific statement in the record wherein Appellant described the claimed invention as requiring the growth of "*de novo*" cardiac muscle or *de novo* arteries, again for good reason. On the other hand, it is clear that Appellant has consistently used the term "new" to describe formation of a cardiac muscle and an artery or portion of an artery that was not in existence before the injection of the stem cells.

Second, it is axiomatic that one does not look to the claims but to the specification for practicing the claimed invention. It is clear from pages 45 and 46 of the specification that Appellant has used the term "new muscle" – not the alleged *de novo* muscle – to describe the formed muscle. It is further clear from the description on pages 53, 54, 56 and 62 (Examples 18, 19 and 36) of the specification that Appellant has given notice to

[1] The Kornowski et al. patent does not constitute a competent prior art reference.

the public by presenting several examples included under the language "new artery." One skilled in the art reading these Examples and the disclosure at pages 45 and 46 would understand that different artery formation mechanisms are described. Contrary to the Examiner's assertion, the various types of new artery formation described in the passages quoted by the Examiner, when viewed in light of the entire specification as a whole, establish the fact that Appellant's invention is not limited to a particular mechanism of artery formation. There is no requirement that Appellant understand the exact scientific theory/principle underlying the discovery that a new artery is formed to obtain a patent.

For the first time, at pages 16 and 31 of the Answer, the Examiner alleged that the Amendment filed February 17, 2004, constituted an intent by Appellant to distinguish the claims over Murry's blood vessel growth from arteries in an integral fashion. Such allegation of Appellant's intent finds no support in the record and is contrary to Appellant's intention, as well as the record itself. Moreover, Appellant notes that the Examiner, at page 16, line 9 of the Answer inaccurately states that Murry "presumably" grew new capillaries from existing arteries. The Examiner does not specifically point to any disclosure or teaching in Murry that supports such erroneous presumption. Moreover, those skilled in the art would understand that the unipotent satellite skeletal muscle cells used by Murry are incapable of forming arterial collaterial blood vessels. Appellant has continuously argued that there is a difference in kind between the unipotent cells of Murry and the pluripotent stem cells of the present invention, which produces different results.

Following the interview of January 6, 2004, the term "new" was added to the claims in light of the suggestion by Examiner Kemmerer's then supervisor, Dr. Yvonne

Eyler, that such amendment would be helpful to distinguish Appellant's heart repair method from that of Murry. Appellant's counsel stated that the claims would be amended to include the words "forming new arteries" rather than "forming arteries" and further pointed out that the specification supported such amendment. Appellant further stated that such amendment would be made to more clearly define differences over Murry and mentioned such distinction in the Amendment filed February 17, 2004. The distinction is that Murry fused skeletal muscle cells onto the heart of a murine model without any teaching of obtaining artery formation or any histological evidence thereof, whereas the present invention promotes growth of a new artery in the heart of a human patient by implanting stem cells to affect repair of a damaged heart. Accordingly, Appellant is surprised that the Examiner belatedly raised a new interpretation of the word "new." Certainly, no indication that the word "new" was understood as now described by the Examiner was raised during the interview by any of the three Examiners attending the interview, later on the record, or by Appellant.

Appellant has continuously argued the patentability of each claim separately, i.e., the patentability of the claims on appeal do not stand or fall together. It is noted that the Examiner did not treat the patentability of each claim in the Answer despite the inaccurate, newly presented assertion at page 16, line 21 to page 17, line 2 of the Answer that pages 7-11 of the Office Action of September 22, 2006 treated each claim separately. A reasonable reading of said pages 7-11 does not support the Examiner's assertion because the Examiner failed to specifically address the enablement of the subject matter set forth in each of the claims on appeal. Rather, the Examiner treated all claims broadly and thus failed to address the enablement of each claim. It is apparent to Appellant, for

example, that the Examiner's determination failed to consider the enablement provided by the specification, as a whole, in regard to claims calling for injecting mononuclear bone marrow stem cells "in a body of a human patient" to repair "a dead portion" (claim 268) or "a damaged portion" (claim 269) of said patient's heart or to claims calling for "locally placing" of "a cell" at a site "adjacent to said dead portion" (claim 270) or "adjacent to said damaged portion" (claim 271). Specifically, the Examiner has failed to provide cogent reasons why, in view of the high level of skill in the medical art coupled with the fact that all of the methods and materials needed to practice the invention are well known, skilled medical professionals apprised of Examples 18, 19, and 36 would not be enabled to practice the methods of the aforesaid claims without resort to undue experimentation. Injection of therapeutic agents via a hypodermic syringe is commonplace in the treatment of disease. Consequently, skilled medical workers require little additional guidance to perform such task, including the injection of stem cells. Thus, there should be no question that claims 268 and 269, which call for the injection of stem cells, are enabled by Appellant's specification. It is evident that in the above-mentioned Office Action of September 22, 2006 and in the Answer, the Examiner, by only broadly treating the specification and claims, erroneously dismissed many relevant portions of the specification related to enablement of the claimed subject matter.

At page 17 of the Answer, the Examiner for the first time asserts that Murry discloses experimentation but does not specifically describe the nature and extent of the alleged experimentation. On the same page, for the first time, the Examiner alleges that Deb "speaks to the lack of enablement of any of the claims that do not require local

administration." Appellant is astonished by the Examiner's newly placed reliance upon Deb because Deb's work is not related to employing cell therapy to treat a diseased heart.

At page 18 of the Answer, the Examiner alleges that Nabel et al. (of record and hereinafter "Nabel") constitutes evidence of non-enablement of the claims. A reading of Nabel clearly shows that there was no objective or attempt made to form a new artery and repair dead or damaged portions of a heart, and thus Nabel is not relevant to the enablement of the claimed invention. Nabel does not disclose using the type of cells called for by the present invention, i.e., pluripotent stem cells of the type contained in bone marrow, and therefore cannot relate to the enablement of treating a dead or damaged portion of a heart by growing new arteries and cardiac muscle.

At pages 20 and 21 of the Answer, in response to Appellant's challenge to identify wherein experimentation is described by Strauer 2002, the Examiner, for the first time, improperly imputes that Strauer 2002 may have conducted, but not reported, failed experiments in an effort to bolster her case. The Examiner fails to provide evidence that such practice is commonplace in the medical field, and for good reason. Such rank speculation does not rise to the level of objective evidence. In regard to the Examiner's statement that Strauer 2002's cell population is critical, it is clear from Strauer 2002 that, at the pages referred to by the Examiner, Strauer 2002 appears to have relied only upon the prior work of others, including the selection of cell population, rather than upon experimentation. See page 1916, right column, paragraph 1 (quoted at page 7 in this paper) in this regard. In any event, Strauer 2002 must speak for itself – and in so speaking – does not disclose experimentation, whether failed or otherwise.

At page 19 of the Answer, the Examiner newly raises the issue of whether sufficient disclosure is present to enable one skilled in the art to practice the well known medical technique relating to the containment of therapeutic agents. Those skilled in the art would recognize that little, if any, containment would be required when injecting therapeutic agents via a hypodermic syringe. In any event, the Examiner did not provide any scientifically sound reasoning or evidence as to why these Examples would not have enabled one skilled in the art to practice the scope of the claimed subject matter. Hence, such comments amount to no more than speculation and are devoid of probative value. Regarding the new issue raised by the Examiner that protocols for the containment systems for genes and cells would not be the same, the Examiner failed to offer any explanation or evidence that liquid therapeutic solutions, whether gene or cell based, would require materially different containment system.

The Examiner on page 20, for the first time, raised a broader issue that, for genes and cells, i.e., "[the] scientific considerations of handling, dosage, carriers, etc. are completely different." Again, the Examiner offered no reasoning or evidence. Any skilled person in the art would be familiar with and readily understand how to handle and administer these materials. In rebuttal thereof, Appellant points to U.S. Patent No. 5,980,887 granted to Isner et al.[2] (attached hereto as Exhibit B), which administers both genes and cells to a human patient to grow an endothelial tissue layer. A reading of such patent does not indicate that materially different protocols are required for genes and cells. It is clear that Appellant's specification treated genes and cells as belonging to a

[2] The claimed invention distinguishes from the Isner et al. patent by injecting global bone marrow stem cells which contain not only endothelial progenitor cells isolated and used by Isner but also hemapoietic progenitors, mesenchymal progenitors, and additional fractions requisite for growth of arteries.

class of compositions for promoting soft tissue growth. There is no requirement that a specification to be enabling has to disclose a distinct embodiment corresponding to each claim at issue. Cf. Union Oil of Cal. v. Atlantic Richfield Co., 208 F3d 989, 997; 54 USPQ 1227, 1232-33 (Fed. Cir. 2000).

At pages 20, 33, and 34 of Answer, the Examiner first alleged that the selection of dosages for cells and genes involves completely different scientific considerations. Again, the Examiner offered no reasoning or evidence in support of such allegation.

Dosage selection is well established to be a matter of common medical practice, dependent upon a number of factors well known in the medical art. Hence, the selection of dosages is considered to be within the skill of the art. In any event, the Examiner for the first time on pages 33 and 34 of the Answer addresses the dosage extrapolation presented in the Amendment filed June 22, 2006. This extrapolation was first presented by Appellant in the withdrawn Appeal Brief of June 9, 2005, as well as in the June 22, 2006 Amendment. Accordingly, the Examiner's statement on page 33, lines 14-17 of the Answer that such argument was presented "for the first time on this record" is patently incorrect. What is correct is that the Answer is the first time on the record that the Examiner has attempted to critically review the evidentiary value of this archived matter. Appellant's extrapolation utilizes conversion charts/formulas for estimating dosages of cells from weights of nucleic acids. Appellant believes that workers skilled in the art armed with such knowledge and coupled with the disclosure in Appellant's specification that nucleic acids and stem cells belong to a class of compositions for promoting growth of soft tissues including organs, such as arteries, and specifically apprised of the disclosures in Examples 19 and 36 that direct injection of cellular nucleic acid into a

patient's heart (Example 19) or into sites in the coronary artery (Example 36) in dosages sufficient to promote new artery growth, would reasonably be led to conclude that injecting stem cells in similar dosages on a weight basis would expectedly provide similar results. The specification on pages 47 and 48 in describing that a patient's own stem cells can promote organ growth through differentiation and morphogenesis would constitute a predictor for one skilled in the art to expect that injection of bone marrow stem cells into the heart muscle (Example 19) would result in new artery and muscle growth. The Examiner has not challenged the calculus of Appellant's molecular weight extrapolation; rather, she hubristically asserts that Appellant's calculus is "simply nonsensical and scientifically unsound." Appellant's conversion is what it is—a straightforward weight conversion for estimating dosages of cells from weights of nucleic acids that has been successfully practiced by medical professionals over some fifty years of cell therapy procedures. The simplicity of such conversion is shown by the fact that conversion tables have been long available in the medical art. In this regard, see the Roche conversion tables contained in Exhibit E which is appended to the Third Supplemental Declaration of Dr. Richard Heuser (attached hereto as Exhibit C) and to the Second Supplemental Declaration Dr. Andrew E. Lorincz (attached hereto as Exhibit D). These declarations were originally filed in Appellant's co-pending patent application Serial No. 10/179,589 to support the reasonableness of converting dosages of nucleic acids to dosages of cells.[3] The Examiner's failure to challenge the basis for the conversion data in the Final Rejection of September 22, 2006 necessitated submission of

[3] Examples 17 and 18 identified in said declarations correspond to Examples 18 and 19, respectively, in the instant specification disclosure.

these Declarations in the instant Reply Brief as rebuttal evidence because the omission of comments regarding the conversions was taken as an acquiescence of its validity.

The Examiner's *ad hominem* criticism of Appellant's conversion fails to adequately give weight to its evidentiary value. Appellant's evidence establishes as a material fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids. It is the Examiner, not Appellant, that advances a complicated argument. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages. The general practice is to employ multiple doses since stem cell overdosing has not proved to be problematic. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant cell therapy. The Board's attention is directed to the express opinions of Drs. Heuser and Lorincz in Paragraphs 5 and 6 of the declarations which validate the reasonableness of Appellant's dosage conversions. Of particular note is their respective Paragraph 6 statements that, "a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis." Lest there be any doubt that Appellant's conversion is scientifically sound, it appears to be more than mere happenstance that the present invention's converted dosage range of 6.25 to 12.5 x 10^6 cells overlaps the Strauer 2002 dosage range of 9 to 28 x 10^6 cells. Accordingly, Appellant submits that the entire specification, including the disclosures in Examples 18, 19, and 36, must be considered when determining whether one skilled in the art would have been able to carry out the steps required to practice the

claimed method of inserting (injecting) cells (stem cells) into the damaged heart of a human patient to promote the growth of an artery and thereby repair said damaged heart.

At page 29 of the Answer, the Examiner incorrectly states that "the broadest claims merely recites 'growth factor'." The Examiner apparently has overlooked the fact that a species requirement was made and that Appellant elected cells. Accordingly, the broadest claims under rejection are cells with the narrowest claims calling for bone marrow stem cells. This is commensurate with Appellant's disclosure of using autologous and allogenic bone marrow stem cells to promote growth of organs, e.g., growth of an artery or heart, by differentiation and morphogenesis (see pages 47 and 48 of the specification). That the Strauer 2002 publication used autologous mononuclear bone marrow cells is totally irrelevant to the question of whether the subject specification provides enablement for the subject matter claimed on appeal.

The Examiner, at page 30 of the Answer, incorrectly states that the specification provides no guidance in regard to selecting a desired site for implanting cells to form new cardiac muscle and new arteries. It is clear from pages 45, 56, and 62 of the specification that the cells are implanted into the heart muscle adjacent a clogged artery. The Examiner's comment that the "adjacent" language recited in claims 270 and 271 is a relative term fails to take into account the context of Appellant's specification, and thus is unreasonable under the facts of this case. To the extent that the "adjacent" language may be considered relative, any doubt as to its intended meaning is clearly described to one skilled in the art in the instant specification.

At pages 35 and 36 of the Answer, the Examiner questions what undisclosed manipulation must be employed by the skilled artisan in order to promote growth of an

artery "at the site of a damaged heart" instead of some other organ in the patient's body. This would be akin to questioning what manipulation would be required of a doctor skilled in IVF implantation after placement of a two-cell embryo in a human female patient in order to promote growth of a viable human organism instead of some other primate organism. The posing of this question is telling because it evinces lack of a basic understanding on the Examiner's part in regard to Appellant's novel contribution to the medical arts. The answer is simple. The skilled medical doctor's manipulation ends with the implantation or reimplantation of the bone marrow stem cells in the heart of the patient in sufficient dosages to affect repair of the dead or damaged portion of the patient's heart. It is clear from the instant specification that the stem cells, without any further intervention by medical personnel, act along predetermined genetic pathways to promote growth of the necessary soft tissues via differentiation and morphogenesis. In addition to the instant specification and the supporting declaration evidence of record, the numerous references of record of other highly skilled workers in the medical art including Orlic, Strauer 2002, Perin, Dohmann, Wollert, etc. (of record) act as confirmation of the claimed results.

At page 29, line 10, the Examiner states that sources of energy are growth factors, relying upon pages 20 and 21 of the specification. A reading of these pages leads one to the conclusion the Appellant did not define energy within the disclosed class of growth factors.

At page 36 of the Answer, the Examiner alleges that the specification does not provide guidance as to how an old material, such as bone marrow stem cells, and methods, such as injection into heart muscle, can be used to achieve growth of a new tissue, such as an artery, which the Examiner characterizes as "remarkable" without conducting actual clinical trials. The Examiner further characterized the claimed result as "incredible" in the absence of having done a single experiment. The Board's attention is directed to the Kornowki patent cited earlier which contains claims directed to such "remarkable" results based upon a prophetic disclosure.

At page 37, lines 6-9 of the Answer, the Examiner states Deb (of record) did not demonstrate repair of dead/damaged heart tissue or growth of a new artery. While this is true, any reader of Deb would understand that Deb, like Nabel, neither sought nor made any attempt to form a new artery and repair dead or damaged portions of a heart. Accordingly, Deb is not relevant to the enablement of the claimed invention.

At page 38, lines 5-7 of the Answer, the Examiner erroneously stated that the specification "...does not provide guidance along the lines of Wollert et al.'s use of large quantities of cells and multiple administration passages to compensate for re-migration problems identified by others in this art." In rebuttal, reference is made to the discussion above regarding extrapolated cell dosages of Example 19 which overlap the dosages disclosed by Strauer 2002 and to page 47 of the instant specification where booster shots are contemplated. In regard to the newly alleged "re-migration" problem, the Examiner has not identified a basis in the present record, including Wollert or any others, for establishing the existence of such a "problem." Perhaps the Examiner is confused by the comments in Strauer 2002 relating to the disadvantage of employing multiple circulation

passages when using IV administration of cells. From the record, it appears that "re-migration" is a nonexistent problem created by the Examiner. Particularly, the Examiner has not explained how local injection of stem cells into a patient's heart would encounter a "re-migration" problem.

The Examiner's statements on pages 38-42 addressing Appellant's objective evidence in the form of the Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz evinces a misunderstanding on the part of the Examiner as to the PTO's burden of proof set forth in In re Oetiker, at 1445, 24 USPQ 2d at 1444; In re Keller, 642 F.2d 413, 208 USPQ 871, (CCPA 1981). Further, the Examiner's summary dismissal of the Appellant's declaration evidence constitutes reversible error. Although enablement is a matter of law, it is based on underlying factual determinations. The Examiner cannot discharge her burden of proof by simply determining that the specification is non-enabling and then summarily dismiss Appellant's declaration evidence submitted in rebuttal because (Answer, page 40, lines 7 and 8) "[i]t appears that the experts relied upon the specification itself, which has been separately addressed." The Board's attention is especially directed to Paragraphs 4-7 of the above-identified Heuser and Lorincz declarations. The question of enablement has to be reviewed anew in view of Appellant's submission of objective evidence proffered in rebuttal to the Examiner's original case of lack of enablement. Since it is axiomatic that enablement must be established at the time of invention, it is entirely proper for Appellant's experts to consider the specification disclosure based on their expertise in the medical art in rendering their expert opinions. Appellant submits that the Heuser and Lorincz declarations establish a material fact supporting the enablement of the claimed

invention, which was not adequately weighed and/or rebutted by the Examiner in considering whether the specification as a whole satisfies the enablement requirement of the statute. Failure to adequately weigh Appellant's declaration evidence in determining whether the claimed invention is enabled by the specification constitutes reversible error. See In re Alton, 76 F.3d 1168, 37 USPQ2d 1578 (Fed. Cir.1996).

Finally, in conclusion, Appellant submits that the underlying theme for lack of enablement relied upon by the Examiner (see Answer, page 28) is that, while the specification discloses using old materials (bone marrow stem cells) and old methods (injection) to achieve the claimed result, there is no guidance provided to one skilled in the art as to how to achieve the claimed results. It is readily apparent from the record that "how to" practice the claimed invention, particularly the injection of an old composition (bone marrow stem cells) into the heart of a human patient at a desired site adjacent a clogged coronary artery and thereby affect new artery growth at the site, is enabled by the protocol set forth in Example 19 which provides guidance sufficient to enable one skilled in the art "how to" to practice the claimed invention. As discussed earlier, no further manipulation is required because once implanted or reimplanted, the bone marrow stem cells follow predetermined genetic pathways via differentiation and morphogenesis to promote growth of the desired tissues (see specification pages 45 - 48). To the extent that the Examiner's position is bottomed on the failure of the specification to set forth an example specifically directed to the elected bone marrow stem cell species, that is not the legal standard for enablement. Union Oil of Cal. v. Atlantic Richfield Co., at 1232-33, supra.

CONCLUSION AND RELIEF SOUGHT

In view of the foregoing, Appellant again urges the Board to reverse the outstanding rejection of claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 under 35 U.S.C. §112, first paragraph, and respectfully requests that the instant application be passed to issue.

Respectfully submitted,

Dated: 03/14/08

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CLAIMS APPENDIX

Claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 are pending in the application, are under final rejection, are being appealed, and are listed below.

Claim 236 A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.

Claim 238 The method of claim 236, further comprising repairing a dead portion of said heart.

Claim 239 The method of claim 236, further comprising repairing a damaged portion of said heart.

Claim 243 The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.

Claim 244 The method of claim 243, wherein said growth factor comprises a cell.

Claim 247 The method of claim 236, wherein said growth factor is placed in said patient by injection.

Claim 250 The method of claim 247, wherein said injection is intramuscular.

Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.

Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.

Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.

Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.

Claim 260 The method of claim 243, wherein said growth factor is locally placed in said body.

Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.

Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.

Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 280 The method of claim 236 further comprising calculating blood flow through said newly grown artery.

Claim 281 The method of claim 238 further comprising calculating blood flow through said newly grown artery.

Claim 282 The method of claim 239 further comprising calculating blood flow through said newly grown artery.

Claim 283 The method of claim 236 further comprising observing said newly grown artery.

Claim 284 The method of claim 238 further comprising observing said newly grown artery.

Claim 285 The method of claim 239 further comprising observing said newly grown artery.

EVIDENCE APPENDIX

1. Orlic et al. publication entitled, "Mobilized bone marrow cells repair the infarcted heart, improving function and survival," (August 28, 2001, PNAS USA, 98:10344-10349) and Orlic et al. publication entitled, "Bone marrow cells regenerate infarcted myocardium," (April 5, 2001, Nature, 410:701-705) cited on page 91 in Appellant's June 22, 2006, Amendment

2. Dohmann et al., 2005 publication in Circulation, 112:521-526, full copy of publication furnished in Examiner's January 24, 2008, Answer

3. Kornowski U.S. Patent No. 7,097,832 (attached hereto as Exhibit A)

4. Isner U.S. Patent No. 5,980,887 (attached hereto as Exhibit B)

5. Third Supplemental Declaration of Richard Heuser filed as Exhibit E of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589 (attached hereto as Exhibit C)

6. Second Supplemental Declaration of Andrew E. Lorincz filed as Exhibit D of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589 (attached hereto as Exhibit D)

COPIES OF ITEMS 1-6

Cited in

EVIDENCE APPENDIX

EVIDENCE APPENDIX

ITEM NO. 1

Orlic publications

Mobilized bone marrow cells repair the infarcted heart, improving function and survival

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Edited by Eugene Braunwald, Partners HealthCare System, Inc., Boston, MA, and approved June 29, 2001 (received for review April 11, 2001)

Attempts to repair myocardial infarcts by transplanting cardiomyocytes or skeletal myoblasts have failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the remaining viable portion of the ventricular wall. The recently discovered growth and transdifferentiation potential of primitive bone marrow cells (BMC) prompted us, in an earlier study, to inject in the border zone of acute infarcts Lin$^-$ c-*kit*^{POS} BMC from syngeneic animals. These BMC differentiated into myocytes and vascular structures, ameliorating the function of the infarcted heart. Two critical determinants seem to be required for the transdifferentiation of primitive BMC: tissue damage and a high level of pluripotent cells. On this basis, we hypothesized here that BMC, mobilized by stem cell factor and granulocyte-colony stimulating factor, would home to the infarcted region, replicate, differentiate, and ultimately promote myocardial repair. We report that, in the presence of an acute myocardial infarct, cytokine-mediated translocation of BMC resulted in a significant degree of tissue regeneration 27 days later. Cytokine-induced cardiac repair decreased mortality by 68%, infarct size by 40%, cavitary dilation by 26%, and diastolic stress by 70%. Ejection fraction progressively increased and hemodynamics significantly improved as a consequence of the formation of 15 × 10^6 new myocytes with arterioles and capillaries connected with the circulation of the unaffected ventricle. In conclusion, mobilization of primitive BMC by cytokines might offer a noninvasive therapeutic strategy for the regeneration of the myocardium lost as a result of ischemic heart disease and, perhaps, other forms of cardiac pathology.

Sudden occlusion of a major coronary artery and acute myocardial ischemia lead to rapid death of myocytes (M) and vascular structures in the supplied region of the ventricle. Despite the demonstration that a subpopulation of cardiac muscle cells is able to replicate (1), and new vessels are formed (2), this regeneration is restricted to the viable myocardium. The loss of M, arterioles, and capillaries in the infarcted area is irreversible, resulting with time in the formation of scarred tissue. For this reason, most experimental and clinical therapies have mainly focused on limiting infarct size. Attempts to replace the necrotic zone of the heart by transplanting cardiomyocytes or skeletal myoblasts (3–7), although successful in the survival of many of the grafted cells, have invariably failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the spared ventricular wall.

The recognition that stem cells, particularly those from the bone marrow, have the capacity to colonize different tissues, proliferate, and transdifferentiate into cell lineages of the host organ (8, 9), prompted us in an earlier study (10) to inject Lin$^-$ c-*kit*^{POS} bone marrow cells (BMC) in the contracting myocardium bordering acute infarcts. Surprisingly, the implanted BMC differentiated into M and coronary vessels ameliorating the function of the injured heart (10). This approach, however, required a surgical intervention that was accompanied by high mortality and a grafting success rate of 40%. Therefore, the identification and utilization of a noninvasive method would be highly desirable. Two main determinants seem to be critical for colonization and transdifferentiation of BMC into a variety of tissues: recent damage and a high number of circulating stem cells (8, 9, 11, 12). On this basis, we hypothesized that a sufficient number of BMC mobilized by stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF; refs. 13 and 14) would home to the infarcted heart and promote cardiac repair. To test this possibility, mice were injected with SCF and G-CSF to increase the number of circulating stem cells from 29 in nontreated controls to 7,200 in cytokine-treated mice (13).

Materials and Methods

Myocardial Infarction (MI) and Cytokines. C57BL/6 male mice at 2 months of age were splenectomized and 2 weeks later were injected s.c. with recombinant rat SCF, 200 μg/kg/day, and recombinant human G-CSF, 50 μg/kg/day (Amgen Biologicals), once a day for 5 days (13, 14). Under ether anesthesia, the left ventricle (LV) was exposed and the coronary artery was ligated (10, 15, 16). SCF and G-CSF were given for 3 more days. Controls consisted of splenectomized infarcted and sham-operated (SO) mice injected with saline. BrdUrd, 50 mg/kg body weight, was given once a day for 13 days before the mice were killed; mice were killed at 27 days. Protocols were approved by New York Medical College.

Echocardiography and Hemodynamics. Echocardiography was performed in conscious mice by using a Sequoia 256c (Acuson, Mountain View, CA) equipped with a 13-MHz linear transducer (15L8). The anterior chest area was shaved and two- dimensional (2D) images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole were obtained (17). Ejection fraction (EF) was derived from LV cross-sectional area in 2D short axis view (17): $EF = [(LVDA - LVSA)/LVDA] \times 100$, where LVDA and LVSA correspond to LV areas in diastole and in systole. Mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), and a microtip pressure transducer (SPR-671; Millar Instruments, Houston) connected to a chart recorder was advanced into the LV for the evaluation of pressures and + and − dP/dt in the closed-chest preparation (10, 15, 16).

Cardiac Anatomy and Infarct Size. After hemodynamic measurements, the abdominal aorta was cannulated, the heart was arrested in diastole with $CdCl_2$, and the myocardium was perfused with 10% (vol/vol) formalin. The LV chamber was filled with fixative at a pressure equal to the *in vivo* measured

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BMC, bone marrow cells; SCF, stem cell factor; G-CSF, granulocyte-colony stimulating factor; LV, left ventricle; EC, endothelial cells; SMC, smooth muscle cells; EF, ejection fraction; SO, sham-operated; LVFW, LV free wall; M, myocyte; MI, myocardial infarction.

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end-diastolic pressure (15, 16). The LV intracavitary axis was measured, and three transverse slices from the base, mid-region, and apex were embedded in paraffin. The mid-section was used to measure LV thickness, chamber diameter, and volume (15, 16). Infarct size was determined by the number of M lost from the left ventricular free wall (LVFW; refs. 18 and 19).

Newly Formed M. The volume of regenerating myocardium was determined by measuring in each of three sections the area occupied by the restored tissue and section thickness. The product of these two variables yielded the volume of tissue repair in each section. Values in the three sections were added, and the total volume of formed myocardium was obtained. Additionally, the volume of 400 M was measured in each heart. Sections were stained with desmin and laminin Abs and propidium iodide (PI). Only longitudinally oriented cells with centrally located nuclei were included. The length and diameter across the nucleus were collected in each M to compute cell volume, assuming a cylindrical shape (18, 19). M were divided in classes, and the number of M in each class was calculated from the quotient of total M class volume and average cell volume (20, 21). The number of arteriole and capillary profiles per unit area of myocardium was measured as described (18, 19).

BrdUrd and Ki67. Sections were incubated with BrdUrd or Ki67 Ab. M were recognized with a mouse monoclonal anti-cardiac myosin, endothelial cells (EC) were recognized with rabbit polyclonal anti-factor VIII, and smooth muscle cells (SMC) were recognized with a mouse monoclonal anti-α-smooth muscle actin. The fractions of M, EC, and SMC nuclei labeled by BrdUrd and Ki67 were obtained by confocal microscopy (10). Nuclei sampled in 11 cytokine-treated mice for BrdUrd were M = 3,541; EC = 2,604; SMC = 1,824; and for Ki67 were M = 3,096; EC = 2,465; SMC = 1,404.

Cell Differentiation. Cytoplasmic and nuclear markers were used; M nuclei, rabbit polyclonal Csx/Nkx2.5, MEF2, and GATA4 Abs (10, 22, 23); cytoplasm, mouse monoclonal nestin (24), rabbit polyclonal desmin (25), cardiac myosin, mouse monoclonal α-sarcomeric actin, and rabbit polyclonal connexin 43 Abs (10); EC cytoplasm, mouse monoclonal flk-1, vascular endothelial (VE)-cadherin, and factor VIII Abs (10, 26, 27); and SMC cytoplasm, flk-1 and α-smooth muscle actin Abs (10, 28). Scar was detected by a mixture of collagen type I and type III Abs.

Statistics. Results are mean ± SD. Significance was determined by the Student's t test and Bonferroni method (16). Mortality was computed with a log-rank test. $P < 0.05$ was significant.

Results

BMC Mobilization by Cytokines Reduces Mortality and Induces Myocardial Repair After Infarction. Given the ability of bone marrow Lin$^-$ c-*kit*^{POS} cells to transdifferentiate into the cardiogenic lineage (10), we used a protocol to maximize their number in the peripheral circulation to increase the probability of their homing to the region of dead myocardium. In normal animals, the frequency of Lin$^-$ c-*kit*^{POS} cells in the blood is only a small fraction of similar cells present in the bone marrow (13, 14). We have documented previously that the cytokine treatment used here promotes a marked increase of Lin$^-$ c-*kit*^{POS} cells in the bone marrow and a redistribution of these cells from the bone marrow to the peripheral blood. This protocol leads to a 250-fold increase in Lin$^-$ c-*kit*^{POS} cells in the circulation (13, 14).

In the current study, BMC mobilization by SCF and G-CSF resulted in a dramatic increase in survival of infarcted mice; with cytokine treatment, 73% of mice (11 of 15) survived 27 days, whereas mortality was very high in untreated infarcted mice (Fig. 1*A*). A large number of animals in this group died from 3 to 6 days after MI and only 17% (9 of 52) reached 27 days ($P < 0.001$). Mice that died within 48 h post-MI were not included in the mortality curve to minimize the influence of the surgical trauma. Infarct size was similar in the cytokine- [64 ± 11% (n = 11)] and saline- [62 ± 9% (n = 9)] injected animals as measured by the number of M lost in the LVFW at 27 days (see Fig. 5, which is published as supplemental data on the PNAS web site, www.pnas.org).

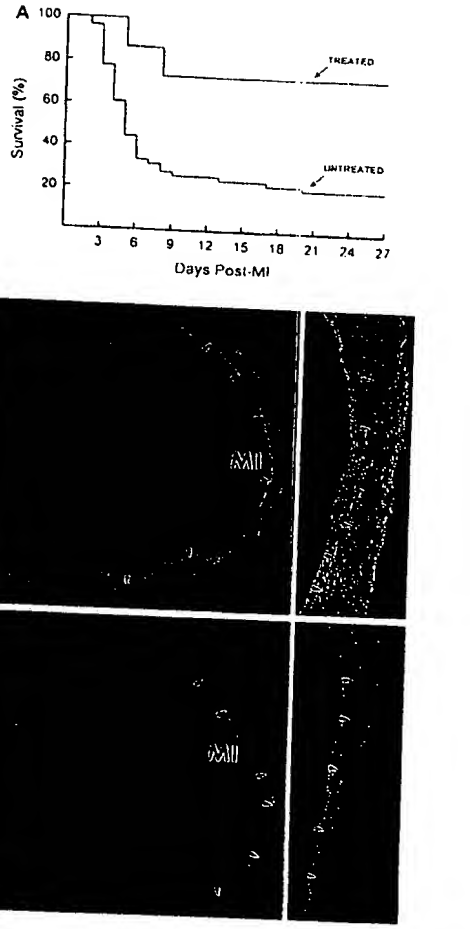

Fig. 1. Mortality and myocardial regeneration. (*A*) Cytokine-treated infarcted mice, n = 15; untreated infarcted mice, n = 52; log-rank test, $P < 0.0001$. (*B*) Large infarct (MI) in a cytokine-treated mouse; forming myocardium (arrowheads) at higher magnification (adjacent panel). (*C*) MI in a nontreated mouse. Healing comprises the entire infarct (arrowheads). Scarring at higher magnification (adjacent panel). Red = cardiac myosin; yellow-green = propidium iodide (PI) labeling of nuclei; blue-magenta = collagen types I and III. (*B* and *C*, ×20; *Insets*, ×80.)

BMC mobilization promoted myocardial regeneration in all 11 cytokine-treated infarcted mice, killed 27 days after surgery (Fig. 1*B*). Myocardial growth within the infarct was also seen in the 4 mice that died prematurely at day 6 (n = 2) and at day 9 (n = 2). Cardiac repair was characterized by a band of newly formed myocardium occupying most of the damaged area. The developing tissue extended from the border zone to the inside of the injured region and from the endocardium to the epicardium of the LVFW. In the absence of cytokines, myocardial replacement was never observed, and healing with scar formation was apparent (Fig. 1*C*). Conversely, only small areas of collagen accumulation were detected in treated mice.

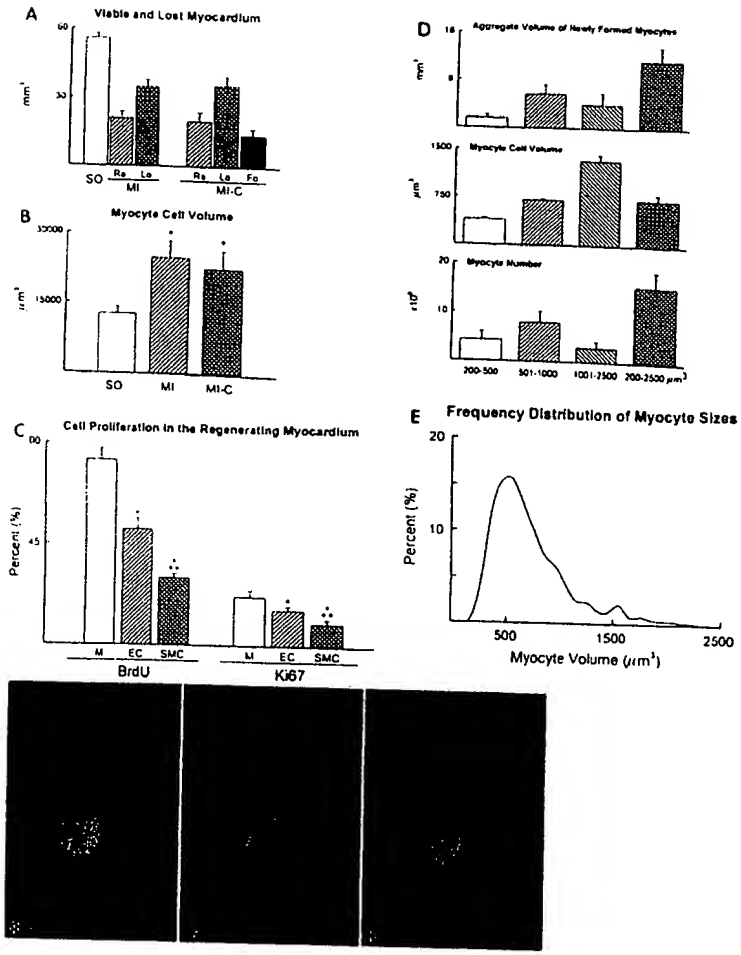

Fig. 2. Myocardial regeneration. (*A*) Remaining viable (Re), lost (Lo), and newly formed (Fo) myocardium in LVFW at 27 days in MI and MI-C; SO, myocardium without infarct. (*B*) Cellular hypertrophy in spared myocardium. (*C*) M, EC, and SMC labeled by BrdUrd and Ki67; $n = 11$. * and **, $P < 0.05$ vs. M and EC. (*D* and *E*) Volume, number ($n = 11$), and class distribution (bucket size, 100 μm^3; $n = 4,400$) of M within the formed myocardium. (*F–H*) Arterioles with TER-119-labeled erythrocyte membrane (green fluorescence); blue fluorescence = propidium iodide (PI) staining of nuclei; red fluorescence = α-smooth muscle actin in SMC. *F*, ×800; *G* and *H*, ×1,200.

BMC Mobilization Partially Restored Myocardial Mass. To quantify the contribution of the developing band to the ventricular mass, we first determined the volume of the LVFW (weight divided by 1.06 g/ml) in each group of mice. These data were 56 ± 2 mm^3 in SO, 62 ± 4 mm^3 (viable FW = 41 ± 3; infarcted FW = 21 ± 4) in infarcted nontreated animals, and 56 ± 9 mm^3 (viable FW = 37 ± 8; infarcted FW = 19 ± 5) in infarcted cytokine-treated mice. These values were compared with the expected values of spared and lost myocardium at 27 days, given the size of the infarct in the nontreated and cytokine-treated animals. From the volume of the LVFW (56 mm^3) in SO and infarct size in nontreated (62%) and treated (64%) mice, it was possible to calculate the volume of myocardium destined to remain (nontreated = 21 mm^3; treated = 20 mm^3) and destined to be lost (nontreated = 35 mm^3; treated = 36 mm^3) 27 days after coronary occlusion (Fig. 2*A*). The volume of newly formed myocardium was detected exclusively in cytokine-treated mice and found to be 14 mm^3 (Fig. 2*A*). Thus, the repair band reduced infarct size from 64% (36 mm^3/56 mm^3 = 64%) to 39% [(36 mm^3 − 14 mm^3)/56 mm^3 = 39%]. Because the spared portion of the LVFW at 27 days was 41 and 37 mm^3 in nontreated and treated mice (see above), the remaining myocardium, shown in Fig. 2*A*, underwent 95% ($P < 0.001$) and 85% ($P < 0.001$) hypertrophy, respectively. Consistently, M cell volume increased 94% and 77% (Fig. 2*B*).

Myocardial Regeneration Is Characterized by Dividing Myocytes and Forming Vascular Structures. Ki67 and BrdUrd were used to evaluate the growth stage of the cells in the regenerating band (Fig. 6 *A–D*, which is published as supplemental data on the PNAS web site). BrdUrd was injected daily between days 14–26 to measure the cumulative extent of cell proliferation while Ki67 was assayed to determine the number of cycling cells at the time of death. Ki67 identifies cells in G_1, S, G_2, prophase, and metaphase, decreasing in anaphase and telophase (10). The percentages of BrdUrd- and Ki67-positive M were 1.6- and 1.4-fold higher than EC, and 2.8- and 2.2-fold higher than SMC, respectively (Fig. 2*C*). The forming myocardium occupied 76 ± 11% of the infarct; M constituted 61 ± 12%, new vessels 12 ± 5%, and other components 3 ± 2%. The band contained 15 × 10^6 regenerating M that were in an active growing phase and had a wide size distribution (Fig. 2 *D* and *E*). EC and SMC growth resulted in the formation of 15 ± 5 arterioles and 348 ± 82 capillaries per mm^2 of new myocardium. Thick wall arterioles with several layers of SMC and luminal diameters of 10–30 μm represented vessels in early differentiation. At times, incomplete perfusion of the coronary branches within the repairing myocardium during the fixation procedure led to arterioles and capillaries containing erythrocytes (Fig. 2 *F–H*). This observation provided evidence that the new vessels were functionally competent and connected with the coronary circulation. Therefore, tissue repair reduced infarct size and M growth exceeded angiogenesis; muscle mass replacement was the prevailing feature of the infarcted heart.

Five cytoplasmic proteins were identified to establish the state

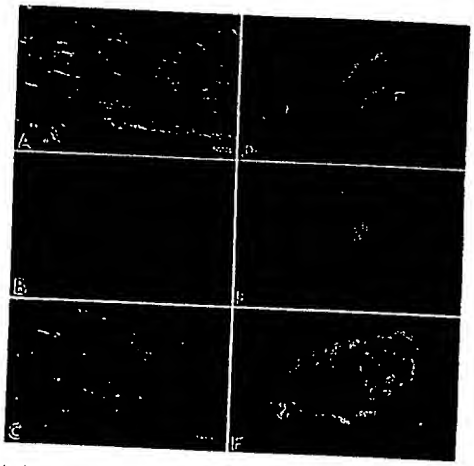

Fig. 3. Markers of differentiating cardiac cells. (*A–F*) Labeling of M by nestin (*A*, yellow), desmin (*B*, red), and connexin 43 (*C*, green); red fluorescence = cardiac myosin (*A* and *C*). (*D* and *E*) Yellow-green fluorescence reflects labeling of EC by flk-1 (arrows, *D*) and VE-cadherin (arrows, *E*); red fluorescence = factor VIII in EC (*D* and *E*). (*F*) Green fluorescence labeling of SMC cytoplasms by flk-1; endothelial lining is also labeled by flk-1; red fluorescence = α-smooth muscle actin; blue fluorescence = propidium iodide (PI) labeling of nuclei. (*A* and *E*, ×1,200; *B* and *F* ×800; *C*, ×1,400; *D*, ×1,800.)

of differentiation of M (10, 24, 25): nestin, desmin, α-sarcomeric actin, cardiac myosin, and connexin 43. Nestin was recognized in individual cells scattered across the forming band (Fig. 3*A*). With this exception, all other M expressed desmin (Fig. 3*B*), α-sarcomeric actin, cardiac myosin, and connexin 43 (Fig. 3*C*). Three transcription factors implicated in the activation of the promoter of several cardiac muscle structural genes were examined (10, 22, 23): Csx/Nkx2.5, GATA-4, and MEF2 (Fig. 7 *A–C*, which is published as supplemental data on the PNAS web site). Single cells positive for flk-1 and VE-cadherin (26, 27), two EC markers, were present in the repairing tissue (Fig. 3 *D* and *E*); flk-1 was detected in SMC isolated or within the arteriolar wall (Fig. 3*F*). This tyrosine kinase receptor promotes migration of SMC during angiogenesis (28). Therefore, repair of the infarcted heart involved growth and differentiation of all cardiac cell populations, resulting in *de novo* myocardium.

Myocardial Repair Improved Anatomical Remodeling and Ventricular Function. Myocardial regeneration attenuated cavitary dilation and mural thinning during the evolution of the infarcted heart *in vivo*. Echocardiographically, LV end-systolic (LVESD) and end-diastolic diameters (LVEDD) increased more in nontreated than in cytokine-treated mice at 9, 16, and 26 days after infarction (Fig. 8 *A* and *B*, which is published as supplemental data on the PNAS web site). Infarction prevented the evaluation of anterior wall systolic thickness (AWST) and anterior wall diastolic thickness (AWDT) . When measurable, posterior wall thickness in systole (PWST) and diastole (PWDT) was greater in treated mice (Fig. 8 *C* and *D*). Anatomically, the wall bordering and remote from infarction was 26% and 22% thicker in cytokine-injected mice (Fig. 8 *E*). BMC-induced repair resulted in a 42% higher wall thickness to chamber radius ratio (Fig. 4*A*). Additionally, tissue regeneration decreased the expansion in cavitary diameter (−14%), longitudinal axis (−5%; Fig. 8 *F* and *G*), and chamber volume (−26%; Fig. 4*B*). Importantly, ventricular mass to chamber volume ratio was 36% higher in treated animals (Fig. 4*C*). Therefore, BMC mobilization that led to proliferation and differentiation of a new population of M and vascular structures attenuated the anatomical variables which define cardiac decompensation.

Measurements of EF during the evolution of infarction and hemodynamics at the time of death showed that repair improved ventricular performance. EF was 48, 62, and 114% higher in treated than in nontreated mice at 9, 16, and 26 days after coronary occlusion, respectively (Fig. 4*D*). In mice exposed to cytokines, contractile function developed with time in the infarcted region of the wall (Fig. 4 *E–M*; Fig. 8 *H–P*). Conversely, LV end-diastolic pressure (LVEDP) increased 76% more in nontreated mice. The changes in LV systolic pressure (not shown), developed pressure (LVDP), and + and − dP/dt were also more severe in the absence of cytokine treatment (Fig. 9 *A–D*, which is published as supplemental data on the PNAS web site). Additionally, the increase in diastolic stress in the zone bordering and remote from infarction was 69–73% lower in cytokine-treated mice (Fig. 4*N*). Therefore, cytokine-mediated infarct repair restored a noticeable level of contraction in the regenerating myocardium, decreasing diastolic wall stress and increasing ventricular performance.

Discussion

On the basis of the results presented above, we conclude that cytokine administration, with the consequent mobilization of BMC into the circulation and, presumably, their translocation to the infarcted portion of the heart, led to a significant magnitude of myocardial repair. Tissue regeneration comprised parenchymal cells and vascular structures. This anatomical restoration was accompanied by a dramatic reduction in post-MI mortality and a remarkable recovery in ventricular performance. Such a high degree of anatomical and functional improvement was accomplished in 100% of the treated animals by using a noninvasive procedure. The importance of these observations for the potential treatment of ischemic heart disease in humans is apparent.

The ability of exogenous BMC to home to the damaged area of the myocardium and differentiate into cells of the cardiogenic lineage, including coronary arterioles and capillaries, was shown previously by local transplantation of Lin$^-$ c-*kit*^{POS} cells into the border zone of an acute infarct (10). However, thoracic surgery and injection of foreign cells were required. Because of the complexity of the protocol, the rate of success with this invasive approach was only 40%. Additionally, this procedure required the availability of syngeneic donors as the source of the transplanted cells. In contrast, the methodology described here succeeded in all cases, eliminated the mortality and morbidity of thoracic surgery and, most importantly, obviated the use of foreign cells with the risk of transmission of infectious agents and the activation of an immunological reaction. Obviously, the generation of true myocardium and coronary vessels is superior to the use of skeletal myoblasts as a replacement for dead cardiac tissue (4, 5, 29). Although skeletal myoblasts survive and differentiate into skeletal muscle when injected into the myocardium, they never become electrically coupled with the rest of the heart because they do not express connexin 43 (4, 5, 29). Moreover, the diastolic properties of skeletal muscle cells are different from those of cardiomyocytes. Similarly, the formation of vessels only severely limits the possibility of complete functional repair after a segmental loss of ventricular mass (30).

Connexin 43 was clearly detectable in the newly formed M derived from BMC at 9 days after implantation (10) and it acquired a more mature pattern of distribution at 27 days. Consistent with the identification of contractile activity in the repairing myocardium, the expression of connexin 43 suggests that operative gap junctions were developed between M. To our knowledge, with the exception of this and our previous report using BMC, none of the published attempts to repair cardiac tissue post-MI have resulted in the production of functional, healthy myocardium. Although not investigated here, this con-

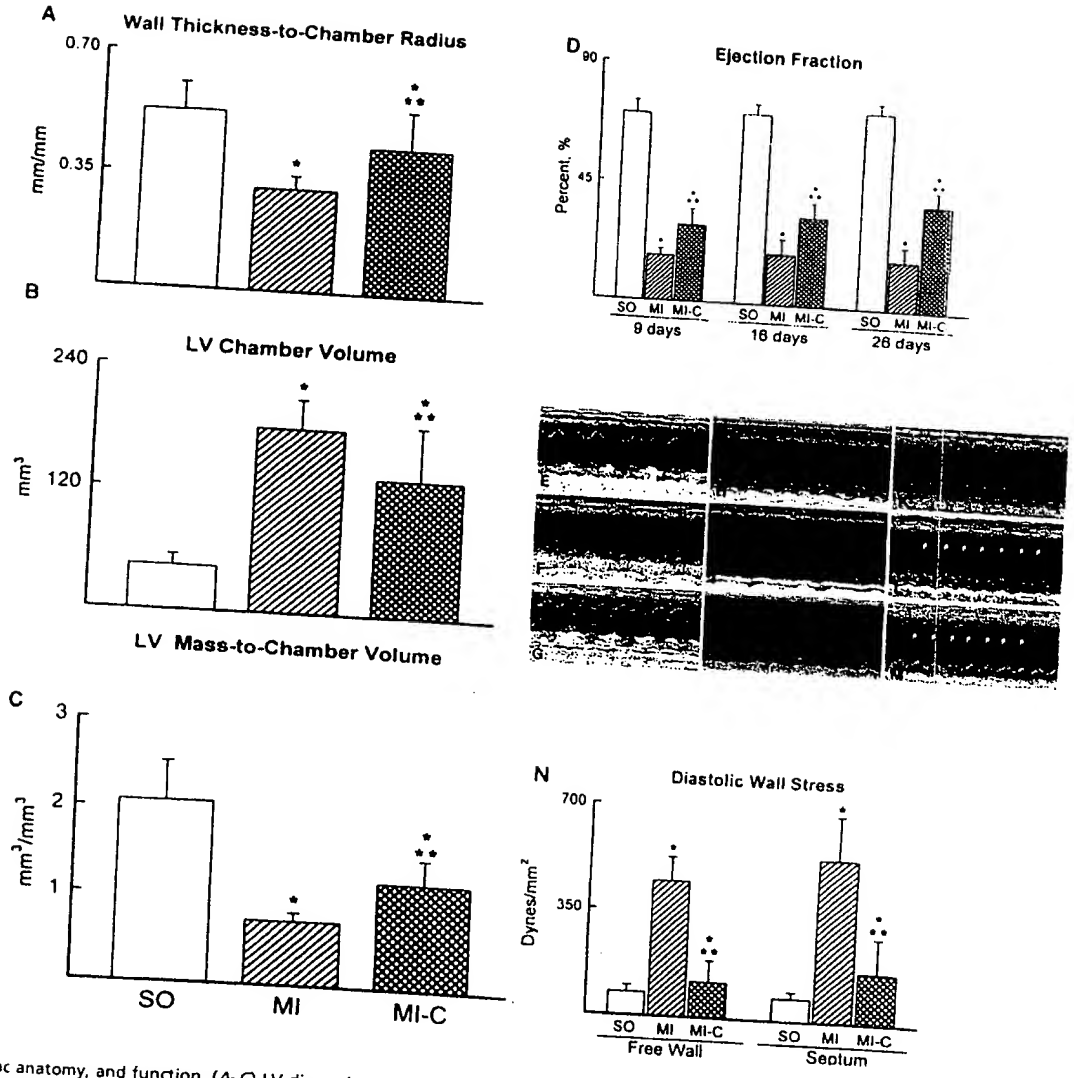

Fig. 4. MI, cardiac anatomy, and function. (*A–C*) LV dimensions at time of death, 27 days after surgery; SO (n = 9), nontreated infarcted (MI, n = 9), and cytokine-treated infarcted (MI-C, n = 10). (*D*) EF by echocardiography (SO, n = 9; MI, n = 9; and MI-C, n = 9). (*E–M*) M-mode echocardiograms of SO (*E–G*), MI (*H–J*), and MI-C (*K–M*); newly formed contracting myocardium (arrows). Detailed echocardiograms are shown in Fig. 8. (*N*) Wall stress, SO (n = 9); MI (n = 8); and MI-C (n = 9). Results are mean ± SD. * and **, $P < 0.05$ vs. SO and MI, respectively.

tention implies that extracellular matrix supporting parenchymal cells and coronary vessels had to be formed. Additionally, scar formation was minimal in the treated animals but this does not exclude the notion that groups of myofibroblasts were present at the edges of the regenerating tissue.

The long-term unfavorable outcome of the infarcted heart is directly related to the initial infarct size that determines the degree of impaired pump function and the magnitude of dilation and wall thinning (31, 32). The changes in cardiac anatomy acutely after infarction, in combination with elevated filling pressure and decreased systolic pressure, induce large increases in diastolic stress and modest increases in systolic stress (18, 31). These structural-functional modifications promote chronic remodeling and the evolution of the myopathy to terminal failure (19, 31). Formation of new myocardium within the infarct attenuated the anatomical alterations, led to chronic increases in EF, and reduced the abnormalities in cavitary pressure, contractility, and loading. Longer intervals after homing of primitive BMC may result in complete repair of the infarcted heart.

Despite the success of the protocol used here, there are questions that we are currently addressing. Administration of SCF and G-CSF mobilizes pluripotent Lin$^-$ c-*kit*^{POS} cells from the bone marrow to the peripheral blood (13). The number of circulating Lin$^-$ c-*kit*^{POS} cells increased 250-fold. Donor BMC injected intravenously home to injured organs including liver (8) and skeletal muscle (33). Because of previous results with Lin$^-$ c-*kit*^{POS} cells (10), we propose that Lin$^-$ c-*kit*^{POS} cells are responsible for cardiac repair. Our results do not provide unambiguous information about the origin of the cells reconstituting the myocardium. It could be argued that cytokine treatment mobilized bone marrow stem cells and resident cardiac stem cells, which together participated in tissue regeneration. We have begun gene-marking studies in an effort to document

the plasticity of adult Lin$^-$ c-*kit*^{POS} bone marrow cells in myocardial repair. This issue could also be addressed by treatment of irradiated animals with cytokines or by transfusing infarcted animals with BMC from syngeneic sex-mismatched donors.

The efficacy of cytokine treatment starting 5 days before MI followed by 3 more days postcoronary occlusion raises the question of the most effective therapeutic window. Additionally, it is not clear whether tissue repair is a result of the homing of BMC to the lesion or whether BMC, once mobilized, nest randomly throughout the organism and only those in the damaged myocardium rapidly proliferate and transdifferentiate. The former possibility is more attractive and supported by the rapid induction of SCF in a number of tissues, including the myocardium (34), in response to injury (34–37). SCF could be responsible for migration, accumulation, and multiplication of primitive BMC in the infarcted zone where they acquire the heart muscle phenotype reaching functional competence.

In conclusion, BMC injected or mobilized to the damaged myocardium behave as cardiac stem cells, giving rise to M, endothelial cells, and smooth muscle cells. Such behavior is no longer surprising given the remarkable plasticity of adult bone marrow stem cells (8–12, 30). New evidence offers clues as to some of the biochemical pathways implicated in this transdifferentiation. The interplay between the signal transduction pathways of bone morphogenetic proteins and the *Wnt* family of genes is responsible for the expression of lineage-determining genes that condition whether a mesodermal precursor cell becomes a blood cell or a cardiac cell (38, 39). During development, the differentiation into a M or a hemopoietic cell is an opposing and mutually exclusive choice. This choice, established by the nature of the cell, seems to be influenced by cues from the environment where the cell resides. Such environmental cues have proven to be difficult to elucidate in the maturing embryo. Surprisingly, as shown here and previously (10, 13), adult BMC remain open to both developmental pathways and readily reprogram themselves in response to the habitat. The absence of hematopoietic islands in the regenerating myocardium as a result of BMC localization is further testimony of the responsiveness of these cells to environmental factors. Thus, this system offers a favorable experimental setting to uncover the biology of this intriguing and potentially clinically useful transdifferentiation.

We believe that the approach presented here might bring myocardial regeneration closer to clinical reality and might also offer the opportunity to uncover the molecular mechanisms involved.

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roughly 300 animals, and given a choice between an optimal concentration of benzaldehyde (1:200 dilution in ethanol) and a lower concentration of diacetyl (1:10,000 dilution in ethanol) in the presence of a uniform field of butanone (1.2 µl per 10-ml plate). Under these conditions more than 95% of wild-type animals prefer benzaldehyde. Animals that accumulated at the diacetyl source were removed and retested under the same conditions to repeat the enrichment. Animals that preferred diacetyl three times were isolated, and their F_3 broods were given a choice between benzaldehyde and diacetyl in the absence of a uniform concentration of butanone. Mutants that could chemotax to benzaldehyde under these conditions were saved. Twenty-seven mutants exhibited discrimination defects that could also be replicated without the diacetyl counterattractant. Mutants were backcrossed twice to wild-type animals.

Genetic mapping of *ky542*

We mapped *ky542* to chromosome II by observing segregation of the discrimination phenotype away from the dominant marker *sqt-1(sc1)* (7/7 isolates). Subsequent mapping was performed by following segregation of the discrimination phenotype with single-nucleotide polymorphisms (SNPs) between the wild-type N2 and CB4856 strains. F_2 progeny of *ky542* × CB4856 crosses were isolated, and populations were generated from each isolate. Each population was tested for butanone/benzaldehyde discrimination. Populations that were homozygous mutant and those that were homozygous wild type were retained, whereas populations that appeared to be heterozygous were discarded. We isolated DNA from each population, and scored SNPs by polymerase chain reaction amplification followed by restriction-enzyme digestion. Using 33 populations, we found that *ky542* mapped between SNPs located on cosmid C01F1 (chromosome II, position −4.5) and cosmid C34F1 (chromosome II, position −2.5).

Laser ablations

AWC neurons were ablated in a wild-type strain that contained an integrated *str-2::GFP* reporter (*kyIs140*) at the L1 or L2 larval stage[17]. The AWC neuron was identified by its characteristic position or by the use of the *str-2::GFP* marker, and then laser irradiated. Ablation was confirmed for AWC^{ON}-ablated animals by looking for *str-2::GFP* expression after all assays had been performed. Single-animal assays were performed on gravid adults as early as the second day after ablation and as late as the fourth day. We assayed the same animals on two or three consecutive days. As many as three consecutive olfactory assays were performed in a single day. For discrimination assays, in which animals were challenged with the same attractant in the presence and absence of saturating odour, animals were allowed to recover between tests for 1 h on a fresh plate with no odours. The order of the assays was randomized on different days. Single-animal assay plates were poured 1 day before the assays and allowed to air dry for 1 h before the assays.

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Bone marrow cells regenerate infarcted myocardium

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Myocardial infarction leads to loss of tissue and impairment of cardiac performance. The remaining myocytes are unable to reconstitute the necrotic tissue, and the post-infarcted heart deteriorates with time[1]. Injury to a target organ is sensed by distant stem cells, which migrate to the site of damage and undergo alternate stem cell differentiation[2–5]; these events promote structural and functional repair[6–8]. This high degree of stem cell plasticity prompted us to test whether dead myocardium could be restored by transplanting bone marrow cells in infarcted mice. We sorted lineage-negative (Lin^-) bone marrow cells from transgenic mice expressing enhanced green fluorescent protein[9] by fluorescence-activated cell sorting on the basis of c-*kit* expression[10]. Shortly after coronary ligation, Lin^- c-kit^{POS} cells were injected in the contracting wall bordering the infarct. Here we report that newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. The developing tissue comprised proliferating myocytes and vascular structures. Our studies indicate that locally delivered bone marrow cells can generate *de novo* myocardium, ameliorating the outcome of coronary artery disease.

Injection of male Lin^-c-kit^{POS} bone marrow cells (see Supplementary Information) in the peri-infarcted left ventricle of female mice resulted in myocardial regeneration. Repair was obtained in 12 out of 30 mice (40%). Failure to reconstitute infarcts was attributed to the difficulty of transplanting cells into tissue contracting at 600 beats per minute. However, an immunological reaction to the histocompatibility antigen on the Y chromosome of the donor bone marrow cells could account for the lack of repair in some of the female recipients. Closely packed myocytes occupied 68 ± 11% of the infarcted region and extended from the anterior to the posterior aspect of the ventricle (Fig. 1a–d). The fraction of endocardial and epicardial circumference delimiting the infarcted area[1,11] did not differ in untreated mice, 78 ± 18% ($n = 8$), or in mice treated with Lin^-c-kit^{POS} cells, 75 ± 14% ($n = 12$), or Lin^-c-kit^{NEG}cells, 75 ± 15% ($n = 11$). New myocytes were not found in mice injected with Lin^-c-kit^{NEG}cells (Fig. 1e).

The origin of the cells in the forming myocardium was deter-

mined by the expression of enhanced green fluorescent protein (EGFP) (Fig. 2; see also Supplementary Information) and the presence of Y chromosome (Supplementary Information). EGFP was restricted to the cytoplasm, whereas Y chromosome was restricted to the nuclei of new cardiac cells. EGFP and Y chromosome were not detected in the surviving portion of the ventricle. EGFP expression was combined with the labelling of proteins specific for myocytes, endothelial cells and smooth muscle cells. This allowed us to identify each cardiac cell type, and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a–c; see also Supplementary Information). The percentage of new myocytes, endothelial cells and smooth muscle cells expressing EGFP was $53 \pm 9\%$ ($n = 7$), $44 \pm 6\%$ ($n = 7$) and $49 \pm 7\%$ ($n = 7$), respectively. These values were consistent with the fraction of transplanted Lin$^-$c-*kit*^{POS} bone marrow cells that expressed EGFP, $44 \pm 10\%$ ($n = 6$). An average $54 \pm 8\%$ ($n = 6$) of myocytes, endothelial cells and smooth muscle cells expressed EGFP in the heart of donor transgenic mice.

To confirm that newly formed myocytes represented maturing cells aiming at functional competence, we examined expression of the myocyte enhancer factor 2 (MEF2), the cardiac specific transcription factor GATA-4 and the early marker of myocyte development Csx/Nkx2.5. In the heart, MEF2 proteins are recruited by GATA-4 to activate synergistically the promoters of several cardiac genes, such as myosin light chain, troponin T, troponin I, α-myosin heavy chain, desmin, atrial natriuretic factor and α-actin[cardiac]. Csx/Nkx2.5 is a transcription factor restricted to the initial phases of

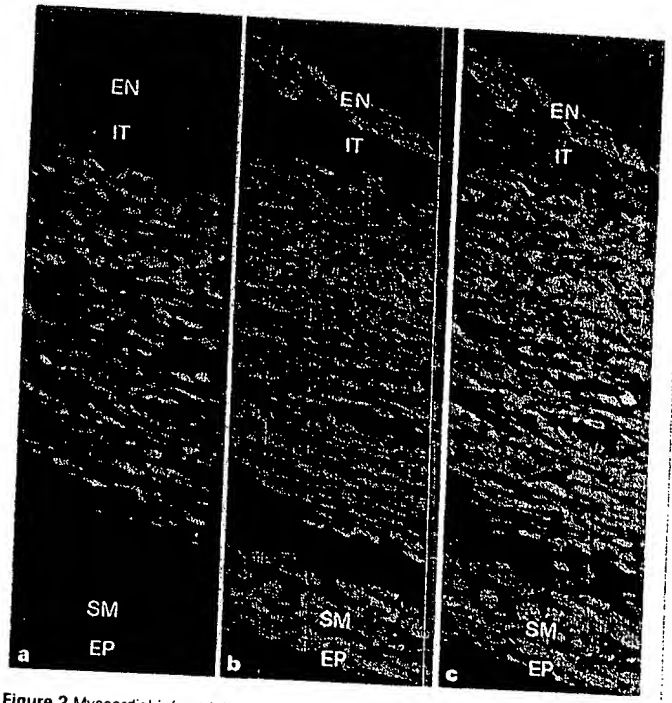

Figure 2 Myocardial infarct injected with Lin$^-$c-*kit*^{POS} cells; myocardium is regenerating from endocardium (EN) to epicardium (EP). **a**, EGFP (green); **b**, cardiac myosin (red); **c**, combination of EGFP and myosin (red–green), and propidium-iodide-stained nuclei (blue). Infarcted tissue (IT) can be seen in the subendocardium, spared myocytes (SM) can be seen in the subepicardium. Original magnification, ×250 (**a**–**c**).

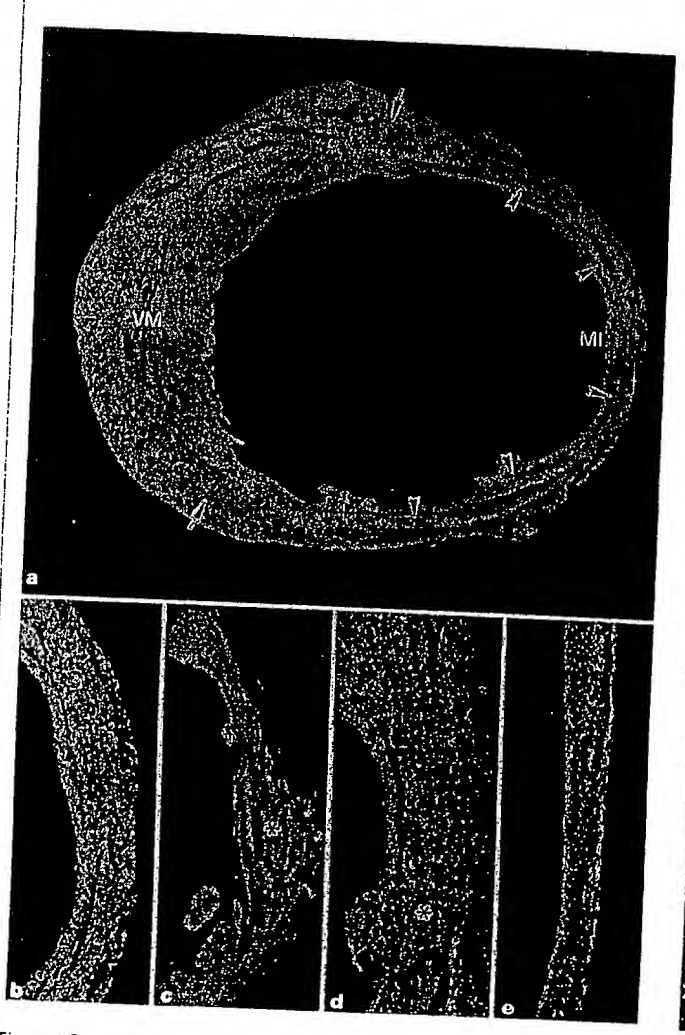

Figure 1 Bone marrow cells and myocardial regeneration. **a**, Myocardial infarct (MI) injected with Lin$^-$ c-*kit*^{POS} cells from bone marrow (arrows). Arrowheads indicate regenerating myocardium; VM, viable myocardium. **b**, Same MI at higher magnification. **c**, **d**, Low and high magnifications of MI injected with Lin$^-$c-*kit*^{POS} cells. **e**, MI injected with Lin$^-$ c-*kit*^{neg} cells; only healing is apparent. Asterisk indicates necrotic myocytes. Red, cardiac myosin; green, propidium iodide labelling of nuclei. Original magnification, ×12 (**a**); ×25 (**c**) ×50 (**b**, **d**, **e**).

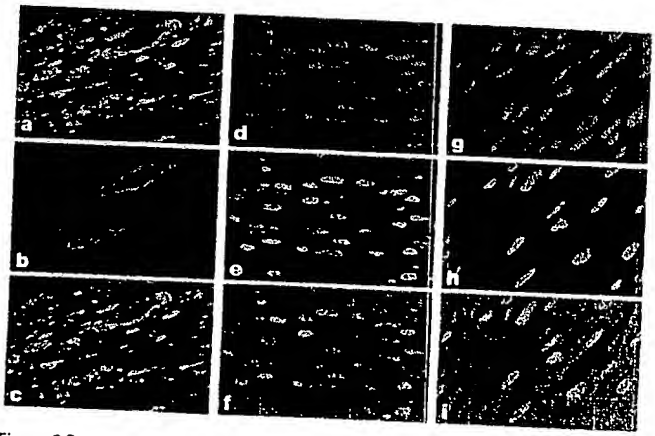

Figure 3 Regenerating myocardium in myocardial infarct injected with Lin$^-$ c-*kit*^{POS} cells; **a**, EGFP (green); **b**, smooth muscle α-actin in arterioles (red); **c**, combination of EGFP and smooth muscle α-actin (yellow–red), and propidium iodide (PI)-stained nuclei (blue). **d**–**i**, MEF2 and Csx/Nkx2.5 in cardiac myosin-positive cells. **d**, **g**, PI-stained nuclei (blue); **e**, **h**, MEF2 and Csx/Nkx2.5 labelling (green); **f**, **i**, cardiac myosin (red), and combination of MEF2 or Csx/Nkx2.5 with PI (bright fluorescence in nuclei). Original magnification, ×300 (**a**–**i**).

myocyte differentiation[12]. In the reconstituting heart, all nuclei of cells labelled with cardiac myosin expressed MEF2 (Fig. 3d–f) and GATA-4 (Supplementary Information), but only 40 ± 9% expressed Csx/Nkx2.5 (Fig. 3g–i).

To characterize further the properties of these myocytes, we determined the expression of connexin 43. This protein is responsible for intercellular connections and electrical coupling through the generation of plasma-membrane channels between myocytes[14,15]; connexin 43 was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells (Fig. 4). These results were consistent with the expected functional competence of the heart muscle phenotype. In addition, myocytes at various stages of maturation were detected within the same and different bands (Fig. 5).

Ki67 is expressed in cycling cells in G1, S, G2 and early mitosis[16], providing a quantitative estimate of the fraction of cells in the cell cycle at the time of observation. 5-Bromodeoxyuridine (BrdU) labelling identifies nuclei in S phase[16,17]; therefore, we injected BrdU for 4–5 days to assess cumulative cell division during active growth (Supplementary Information). Proliferation of myocytes was 93% ($P < 0.001$) and 60% ($P < 0.001$) higher than that of endothelial cells, and 225% ($P < 0.001$) and 176% ($P < 0.001$) higher than that of smooth muscle cells, when measured by BrdU and Ki67, respectively (BrdU: myocytes 36 ± 8%; endothelial cells 19 ± 5%; smooth muscle cells 11 ± 2%; Ki67: myocytes 19 ± 3%; endothelial cells 12 ± 3%; smooth muscle cells 7 ± 2%; $n = 8$ in all cases). Dividing myocytes were small with partially aligned myofibrils, resembling late fetal/neonatal cells; 40–50% of the Ki67- or BrdU-positive cells expressed EGFP.

Cell differentiation caused a loss of c-*kit* surface receptors. We observed only two undifferentiated cells showing c-*kit* on the cell membrane in the subendocardium of the infarcted wall. These c-*kit*-labelled cells were in proximity but not within the regenerating band. They expressed EGFP, confirming their origin from the transplant (Supplementary Information).

To determine whether developing myocytes derived from the Lin$^-$c-*kit*^{POS} cells had an impact on function, we obtained haemodynamic parameters before death. Results from infarcted mice non-injected or injected with Lin$^-$c-*kit*^{NEG} cells were combined. In comparison with sham-operated mice, the infarcted groups exhibited indices of cardiac failure (Fig. 6a). In mice treated with Lin$^-$c-*kit*^{POS} cells, left ventricular (LV) end-diastolic pressure (LVEDP) was 36% lower, and developed pressure (LVDP), LV + d*P*/d*t* and LV − d*P*/d*t* were 32%, 40% and 41% higher, respectively.

Locally transplanted Lin$^-$c-*kit*^{POS} bone marrow cells have a high capacity for cardiac tissue differentiation. Here, they led to the formation of new myocytes, endothelial cells and smooth muscle cells generating *de novo* myocardium, inclusive of coronary arteries, arterioles and capillaries. The partial repair of the infarcted heart implies that the transplanted cells responded to signals from the injured myocardium that promoted their migration, proliferation and differentiation within the necrotic area of the ventricular wall (Fig. 6b). These differentiating myocytes expressed nuclear and cytoplasmic proteins typical of cardiac tissue. The presence of connexin 43 points to cellular coupling and functional competence of the restored myocardium (Fig. 6b). With postnatal maturation, stem cell function was assumed previously to be restricted to cell lineages present in the organ from which they are derived. However, this limitation in stem cell differentiation potential has been challenged by studies showing that bone marrow and neural stem cells can produce many cell types[4,5,18–20]. We report, for the first time, that a subpopulation of primitive bone marrow cells regenerate myocardium *in vivo*, replacing dead tissue.

Haematopoietic stem cells (HSCs), neural-crest-derived melanoblasts and primordial germ cells express c-*kit* on their cell membrane. These primitive cells migrate during fetal development,

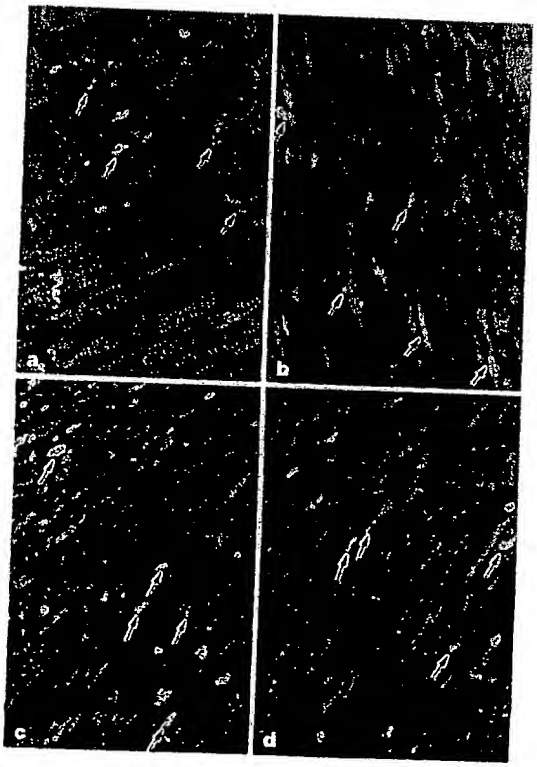

Figure 4 Myocardial repair and connexin 43. **a**, Border zone; **b–d**, regenerating myocardium. Shown are connexin 43 (yellow–green; arrows indicate contacts between myocytes) and α-sarcomeric actin (red), and PI-stained nuclei (blue). Original magnification, ×500 (**a**), ×800 (**b–d**).

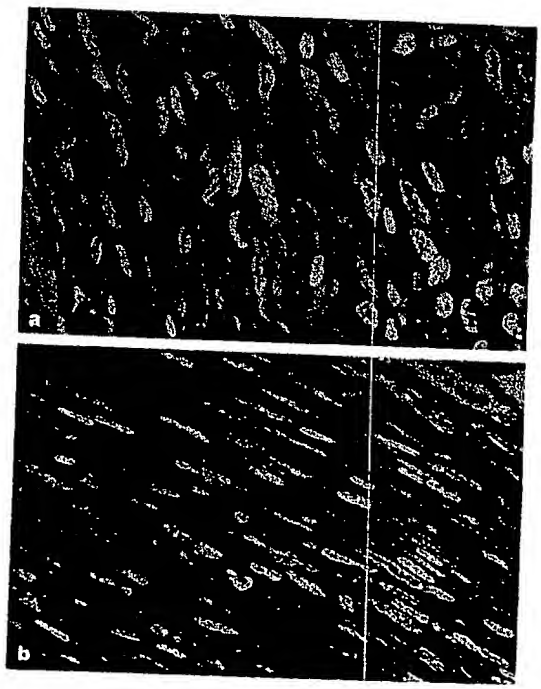

Figure 5 Myocardial infarcts injected with Lin$^-$c-*kit*^{POS} cells: regenerating myocytes. Shown are cardiac myosin (red), and propidium-iodide-labelled nuclei (yellow–green) Original magnification, ×1,000 (**a**); ×700 (**b**).

homing to the yolk sac and liver. Both of these organs are positive for messenger RNA encoding stem cell factor (SCF), the ligand for c-*kit*[21]. It is thought that membrane-bound SCF mediates the migration of HSC and other primitive cells to their target organs[22]. The fetal and neonatal hearts are positive for SCF transcripts[21] and, although it is not clear whether adult heart cells generate SCF, the c-*kit*/SCF pathway might be the mechanism by which, in our conditions, transplanted Lin$^-$c-*kit*^{POS} cells migrated from the site of injection to the infarcted myocardium.

When a stem cell divides, two daughter cells are formed; these may maintain stem cell properties or become differentiating cells[23] that multiply much more rapidly than stem cells[24]. The Lin$^-$c-*kit*^{POS} cells in these transplants produced the three main cell types of the heart: myocytes constituted the predominant and most active growth component of the regenerating myocardium; endothelial and smooth muscle cells were fast growing but were smaller fractions of the developing tissue. Our observations are difficult to compare with those obtained in the cryo-injured rat heart after injecting cultured myocytes derived from mesenchymal bone marrow cells[25]. Formation of myotubules *in vitro* was required for successful transplantation in that study[25], which contrasts with our results. Cryo-injury has no human counterpart. It constitutes an unusual damage with an intact coronary circulation. This may be why only a few endothelial cells were possibly linked to the original culture system[25] and smooth muscle cells were not detected. Also at variance with our data is the fact that there was no replacement of damaged myocardium with functioning tissue.

Coronary heart disease accounts for 50% of all cardiovascular deaths and nearly 40% of the incidence of heart failure. The current findings have provided compelling evidence that our approach has relevant implications for human disease. Locally delivered primitive bone marrow cells promoted successful treatment of large myocardial infarcts after the completion of ischaemic cell death. This therapeutic intervention reduced the infarcted area and improved cardiac haemodynamics. Infarct size is a major determinant of morbidity and mortality, as massive infarcts affecting 40% or more of the left ventricle in patients are associated with intractable cardiogenic shock or the rapid development of congestive heart failure[1]. In the past, recovery of cardiac function has been fully dependent on the growth of the remaining non-infarcted portion of the ventricle. However, the hypertrophied infarcted heart undergoes progressive deterioration, leading to a dilated myopathy, terminal failure and death[1]. Transplanted Lin$^-$c-*kit*^{POS} bone marrow cells have the capability of regenerating acutely significant amounts of contracting myocardium. This new form of repair can improve the immediate and long-term outcome of ischaemic cardiomyopathy. □

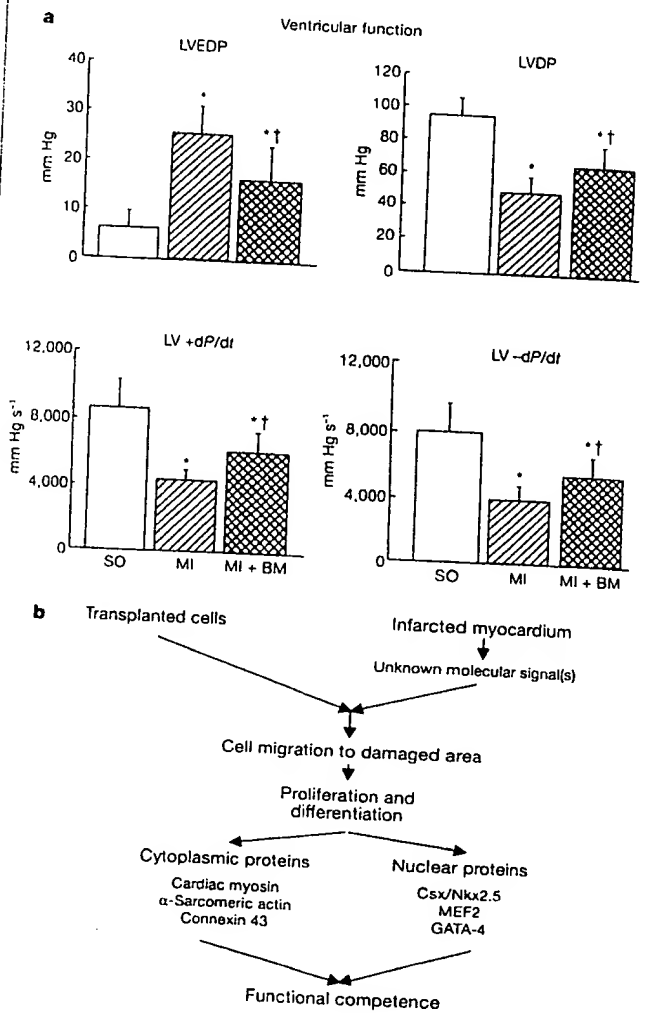

Figure 6 Postulated mechanism of myocardial regeneration and its effect on ventricular function. **a**, Effects of myocardial infarction (MI) on left ventricular end-diastolic pressure (LVEDP), developed pressure (LVDP), LV+ d*P*/d*t* (rate of pressure rise) and LV− d*P*/d*t* (rate of pressure decay). Results are from sham-operated mice (SO, $n = 11$), mice non-injected with Lin$^-$c-*kit*^{POS} cells (MI; $n = 5$ injected with Lin$^-$c-*kit*^{NEG} cells; $n = 6$ non-injected), and mice injected with Lin$^-$c-*kit*^{POS} cells (MI+BM, $n = 9$). Values are mean ± s.d. $^*P < 0.05$ versus SO; $^\dagger P < 0.05$ versus MI. **b**, Proposed scheme for Lin$^-$c-*kit*^{POS} cell differentiation in cardiac muscle and functional implications.

Methods

Lin$^-$c-*kit*^{POS} cells

We collected bone marrow from the femurs and tibias of male transgenic mice expressing EGFP[9]. Cells were suspended in PBS containing 5% fetal calf serum (FCS) and incubated on ice with rat anti-mouse monoclonal antibodies specific for the following haematopoietic lineages: CD4 and CD8 (T lymphocytes), B-220 (B lymphocytes), Mac-1 (macrophages), GR-1 (granulocytes) (all Caltag Laboratories) and TER-119 (erythrocytes) (Pharmingen). Cells were then rinsed in PBS and incubated for 30 min with magnetic beads coated with goat anti-rat immunoglobulin (Polysciences). Lineage-positive cells were removed by a biomagnet and the 10% remaining lineage-negative (Lin$^-$) cells were stained with ACK-4-biotin (anti-c-*kit* monoclonal antibody). Cells were rinsed in PBS, stained with streptavidin-conjugated phycoerythrin (SA-PE) (Caltag) and sorted by FACS using a FACSVantage instrument (Becton Dickinson). We excited EGFP and ACK-4-biotin-SA-PE at a wavelength of 488 nm. The Lin$^-$ cells were sorted as c-*kit*-positive (c-*kit*^{POS}) and c-*kit*-negative (c-*kit*^{NEG}) with a 1–2 log difference in staining intensity. The c-*kit*^{POS} cells were suspended at a concentration of 3×10^4 to 2×10^5 cells in 5 μl of PBS, and the c-*kit*^{NEG} cells were suspended at a concentration of 5×10^4 to 5×10^5 in 5 μl of PBS[10].

Myocardial infarction

Myocardial infarction was induced in female C57BL/6 mice at 2 months of age as described[11]; 3–5 h after infarction, the thorax was re-opened and 2.5 μl PBS containing Lin$^-$c-*kit*^{POS} cells were injected in the anterior and posterior aspects of the viable myocardium bordering the infarct. Infarcted mice that were not injected or injected with Lin$^-$c-*kit*^{NEG} cells and sham-operated mice were used as controls. All animals were killed 9 ± 2 days after surgery. Protocols were approved by an institutional review board.

Ventricular function

Mice were anaesthetized with chloral hydrate (400 mg per kg (body weight), intraperitoneally (i.p.)), and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671; Millar) for the measurements of LV pressures, and LV + and LV− d*P*/d*t* in the closed-chest preparation. The abdominal aorta was cannulated, the heart was arrested in diastole, and the myocardium was perfused retrogradely with 10% buffered formalin[11,26]. Three tissue sections, from the base to the apex of the left ventricle, were stained with haematoxylin and eosin. At 9 ± 2 days after coronary occlusion, the infarcted portion of the ventricle was easily identifiable grossly and histologically (see Fig. 1a). The lengths of the endocardial and epicardial surfaces delimiting the infarcted region, and the endocardium and epicardium of the entire left ventricle, were measured in each section. Subsequently, their quotients were computed to yield the average infarct size in each case. This was accomplished at ×4 magnification with an image analyser connected to a microscope[11].

Cell proliferation and EGFP detection

To establish whether Lin$^-$c-*kit*^{POS} cells resulted in myocardial regeneration, we administered BrdU (50 mg per kg (body weight), i.p.) to the animals daily for 4–5 consecutive days before death. Sections were incubated with anti-BrdU antibody, and BrdU labelling of cardiac cells was measured[19]. Moreover, expression of Ki67 in nuclei was evaluated by treating samples with a rabbit polyclonal anti-mouse Ki67 antibody (Dako). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was used as secondary antibody. EGFP was detected with a rabbit polyclonal anti-GFP (Molecular Probes). Myocytes were recognized with a mouse monoclonal anti-cardiac myosin heavy chain (MAB 1552; Chemicon) or a mouse monoclonal anti sarcomeric α-actin (clone 5C5; Sigma), endothelial cells with a rabbit polyclonal anti-human factor VIII (Sigma) and smooth muscle cells with a mouse monoclonal anti-smooth-muscle α-actin (clone 1A4; Sigma). Nuclei were stained with propidium iodide, 10 μg ml^{-1} (refs 27, 28). We determined the percentages of myocyte (M), endothelial cell (EC) and smooth muscle cell (SMC) nuclei labelled by BrdU and Ki67 by confocal microscopy. This was accomplished by dividing the number of nuclei labelled by the total number of nuclei examined. Numbers of nuclei sampled in each cell population were as follows. BrdU labelling: M, 2,908; EC, 2,153; SMC, 4,877. Ki67 labelling: M, 3,771; EC, 4,051; SMC, 4,752. Numbers of cells counted for EGFP labelling: M, 3,278; EC, 2,056; SMC, 1,274. We determined the percentage of myocytes in the regenerating myocardium by delineating the area occupied by cardiac-myosin-stained cells and dividing this by the total area represented by the infarcted region in each case.

Y chromosome

For the fluorescence *in situ* hybridization assay, we exposed sections to a denaturing solution containing 70% formamide. After dehydration with ethanol, sections were hybridized with the DNA probe CEP Y (satellite III) Spectrum Green (Vysis) for 3 h (ref. 29). Nuclei were stained with propidium iodide.

Transcription factors and connexin 43

Sections were incubated with rabbit polyclonal anti-MEF2 (C-21; Santa Cruz), rabbit polyclonal anti-GATA-4 (H-112; Santa Cruz), rabbit polyclonal anti-Csx/Nkx2.5 (obtained from Dr S. Izumo) and rabbit polyclonal anti-connexin 43 (Sigma). We used FITC-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody[30].

Statistical analysis

Results are presented as means ± s.d. Significance between two measurements was determined by Student's *t*-test, and in multiple comparisons was evaluated by the Bonferroni method. Values of $P < 0.05$ were considered significant.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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CaT1 manifests the pore properties of the calcium-release-activated calcium channel

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The calcium-release-activated Ca^{2+} channel, I_{CRAC}[1–3], is a highly Ca^{2+}-selective ion channel that is activated on depletion of either intracellular Ca^{2+} levels or intracellular Ca^{2+} stores. The unique gating of I_{CRAC} has made it a favourite target of investigation for new signal transduction mechanisms; however, without molecular identification of the channel protein, such studies have been inconclusive. Here we show that the protein CaT1 (ref. 4), which has six membrane-spanning domains, exhibits the unique biophysical properties of I_{CRAC} when expressed in mammalian cells. Like I_{CRAC}, expressed CaT1 protein is Ca^{2+} selective, activated by a reduction in intracellular Ca^{2+} concentration, and inactivated by higher intracellular concentrations of Ca^{2+}. The channel is indistinguishable from I_{CRAC} in the following features: sequence of selectivity to divalent cations; an anomalous mole fraction effect; whole-cell current kinetics; block by lanthanum; loss of selectivity in the absence of divalent cations; and single-channel conductance to Na^+ in divalent-ion-free conditions. CaT1 is activated by both passive and active depletion of calcium stores. We propose that CaT1 comprises all or part of the I_{CRAC} pore.

EVIDENCE APPENDIX

ITEM NO. 2

Dohmann publication

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Heart Failure

Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

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Abstract

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Background— Cell-based therapies for treatment of ischemic heart disease are currently under investigation. We previously reported the results of a phase I trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease. The current report focuses on postmortem cardiac findings from one of the treated patients, who died 11 months after cell therapy.

Methods and Results— Anatomicopathologic, morphometric, and immunocytochemical findings from the anterolateral ventricular wall (with cell therapy) were compared with findings from the interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in the cell-injected areas. Capillary density was significantly higher ($P<0.001$) in the anterolateral wall than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric α-actinin, actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward adjacent bundles of cardiomyocytes.

Conclusions— Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle.

Key Words: angiogenesis • stem cells • heart failure • revascularization • ischemia

Introduction

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The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium's limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells[1,2] has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.[3–6]

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.[7,8] Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.[9] We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

Case Report

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The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis

and resultant episodes of chronic tonic-clonic seizures. His risk factors for coronary artery disease included diabetes mellitus type II, hypertension, and hypercholesterolemia.

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▾ References

The patient's functional capacity was evaluated at baseline by means of a ramp treadmill protocol[10] with a peak maximal oxygen consumption ($\dot{V}O_2max$) of 15.9 mL · kg^{-1} · min^{-1} and a workload of 4.51 metabolic equivalents (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient's coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of $3x10^7$ ABMM cells (the Table) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,[11,12] the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

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Phenotype and Functional Characterization of $3x10^7$ Cells Injected via a Transendocardial Route*

Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient's medications after cell therapy. There was no change in the global ejection fraction or left

ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the $\dot{V}O_2$max increased from 15.8 to 25.2 mL · kg^{-1} · min^{-1}, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the $\dot{V}O_2$max reached 31.6 mL · kg^{-1} · min^{-1}, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient's cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

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After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII–related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α-actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako's EnVision+ System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α-actin, sarcomeric actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, $F(ab)_2$ fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1‰ solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, MediaCybernetics) of randomly selected fields in sections stained with hematoxylin and reacted with antibody for factor VIII–related antigen (n=108) and randomly selected fields in sections reacted with antibodies for smooth muscle α-actin (n=96). Transverse sections of capillaries identified by staining

for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α-actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII–stained slides or the number of capillaries containing pericytes in α-smooth-muscle-actin–stained slides. Larger vessels identified by a continuous wall of smooth muscle actin–positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if P was <0.05.

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at ×100) of factor VIII–stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if P was <0.05.

Results

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Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P<0.0001$) (Figure 1A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.

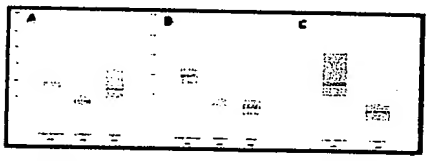

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Figure 1. Number of capillaries per mm^2 in anterolateral, posterior, and septal walls of studied heart. A, Anti-factor VIII–associated antigen counterstained with hematoxylin. B, Anti-smooth muscle α-actin antigen counterstained with hematoxylin. C, Capillaries reacted with anti-factor VIII–associated antigen inside fibrotic areas only in anterolateral and posterior walls. (n=108 microscope fields for A; 96 microscope fields for B; and 40 microscopic fields for C.) Differences were statistically significant among all groups in pairwise comparisons ($P<0.05$, Newman-Keuls method) for A and B. Differences were significantly different ($P<0.05$) between anterolateral and posterior walls in Mann-Whitney rank-sum test for C.

The density of capillaries that contained smooth muscle α-actin–positive cells within their walls was also assessed (Figure 1B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P<0.0001$). Larger vessels identified by a continuous wall of smooth muscle α-actin–positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P<0.0001$) (Figure 1C).

Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the

cardiomyocyte layer and lacked well-defined limits, unlike the fibrotic areas observed in the posterior wall (Figure 2C). No fibrotic tissue was seen in the central area of the septal wall (Figure 2D).

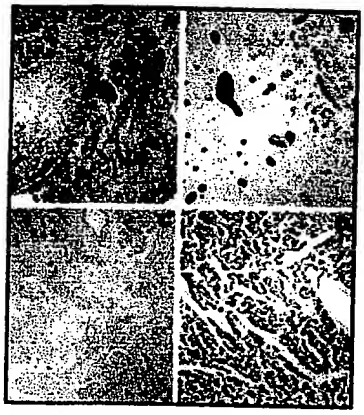

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Figure 2. Gomori trichrome stain of anterolateral (A, B), posterior (C), and septal (D) walls. Increased vascular tree is present in B. Original magnification is x40 in A, B, and D; x100 in C.

Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

Immunocytochemistry Findings

Immunocytochemical labeling of factor VIII–associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal (Figure 3A), and anterolateral (Figure 3B) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle α-actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer (Figure 3C) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells (Figure 3D). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix (Figure 3E). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes (Figure 4A). These cells frequently formed an extensive network that permeated the fibrotic matrix and the

interstitial space among cardiomyocytes (Figure 4B).

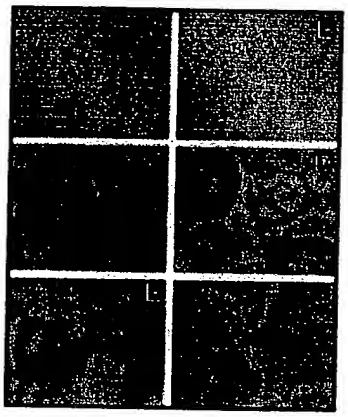

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Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E). Ki67 reactivity was rarely present in perivascular cells of anterolateral wall (F). Original magnification x40 in A and B, x400 in C and D, and x1000 in E and F.

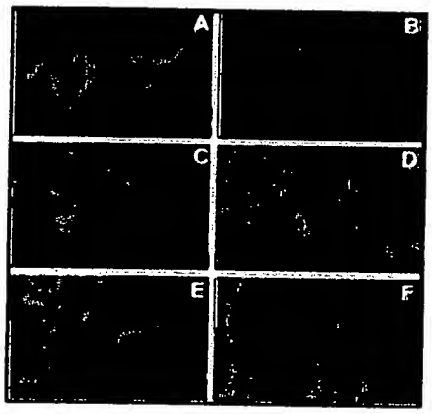

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Figure 4. Anterolateral wall that received cell injection therapy. A and B, Immunostaining for vimentin depicted positive reaction in vascular wall and in fibroblastoid interstitial cells. C and D, Immunostaining for desmin showed small groups of intensely reactive cells between blood vessels and cardiomyocytes (C) and small cells inside cardiomyocyte bundles with typical striated cytoskeleton (D). E and F, Immunostaining for troponin showed positive reaction in all mural cells of medium-sized blood vessel. Original magnification is x1000 in A and F; x400 in B–E.

Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some

of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vàscular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

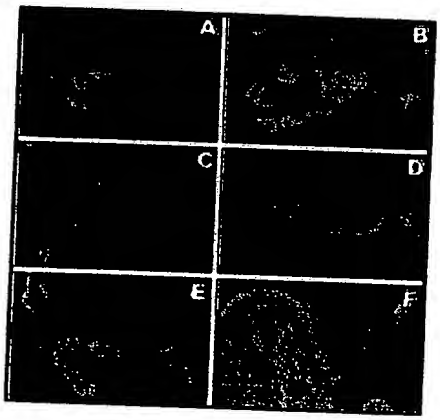

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Figure 5. Anterolateral wall that received cell injection therapy. A, Immunostaining for troponin depicted small cardiomyocyte-like cells with intense reaction in peripheral cell area. B–F, Immunostaining for sarcomeric actinin depicted reactivity in mural cells of blood vessel (B–E) and isolated cells among cardiomyocytes with actinin organization similar to that of sarcomeres (E, F). Original magnification is x400 in A and F; x1000 in B–E.

Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes

could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

Discussion

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Accumulating evidence from both experimental animal studies[4–6] and human trials[7–9] indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure's safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle -actin–positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.[13–15] Endothelial cells can result from bone marrow–derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).[16] Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.[17] In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and their distal gliding on the abluminal side of the growing blood vessel's basement membranes.[13] The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.[18] Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First,

although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforedescribed data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall's improvement in perfusion as assessed by SPECT, whereas all other walls remained unchanged.

Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, troponin, and sarcomeric actinin).

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Acknowledgments

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assistance with statistical questions.

Footnotes

*Drs Dohmann and Perin are coprincipal investigators.

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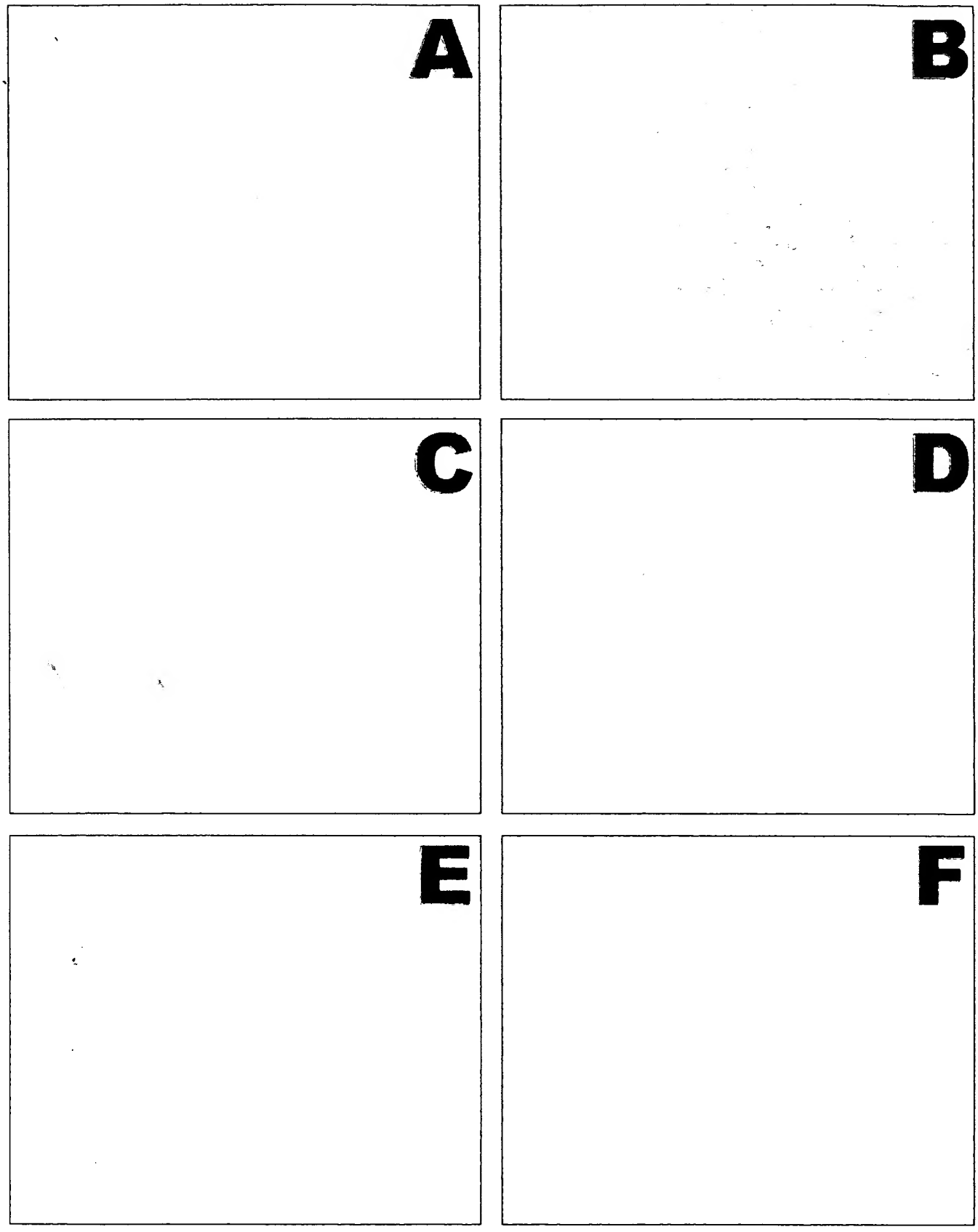
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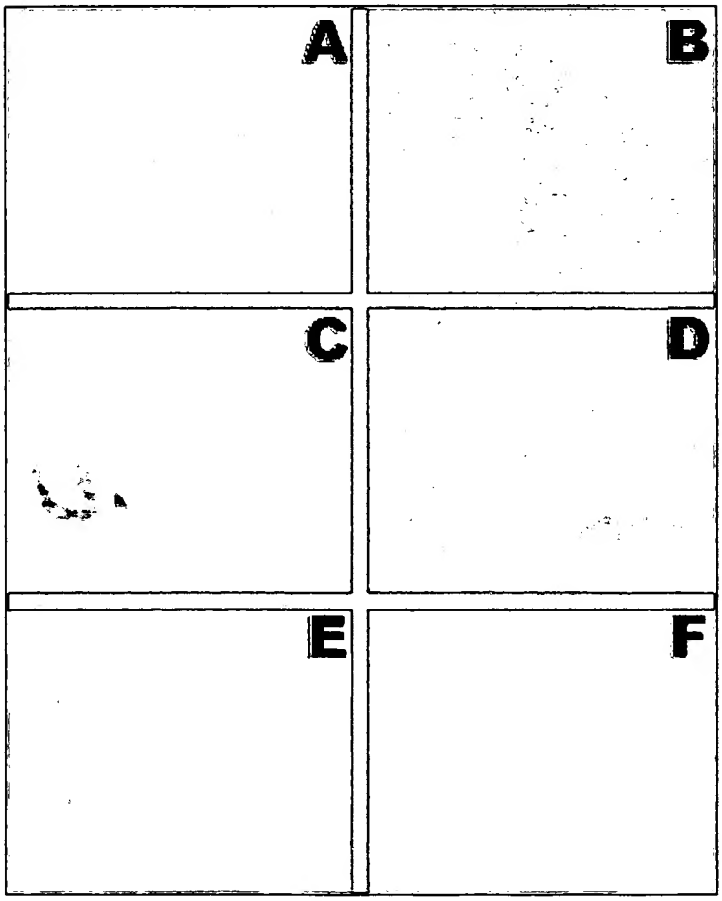

Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E).

EVIDENCE APPENDIX

ITEM NO. 3

Kornowski U.S. Patent No. 7,097,832
(Exhibit A)

EXHIBIT A

Kornowski U.S. Patent No. 7,097,832

EVIDENCE APPENDIX

ITEM NO. 4

Isner U.S. Patent No. 5,980,887
(Exhibit B)

EXHIBIT B

Isner U.S. Patent No. 5,980,887

EVIDENCE APPENDIX

ITEM NO. 5

Third Supplemental Declaration
Of Richard Heuser
(filed in co-pending application SN10/179,589)
(Exhibit C)

EXHIBIT C

3rd Supplemental Declaration of Richard Heuser filed as Exhibit E of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia	)	
	)	Group Art Unit: 1646
Serial No.: 10/179,589	)	
	)	Examiner: Daniel C. Gamett
Filed: June 25, 2002	)	
	)	
For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS	)	

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.

2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Third Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

> ...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20 line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Third Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Third Supplemental Declaration Exhibit C.

6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Third Supplemental Declaration Exhibit E. Note that in two of

the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Third Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 4/20/07

Richard Heuser

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT A

MARCH 7, 2007 OFFICE ACTION Paragraph 11, pages 7-9

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6, and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo, ex vivo,* or *in vitro*.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

ADDITIONAL DISCLOSURES

EXHIBIT C

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1– 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEFG165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30^{th} day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo.* Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8–17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT D

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250 x 10^6 pg and 500 x 10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250 x 10^6 and 500 x 10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25 x 10^6 and 12.5 x10^6, respectively.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT E

PUBLICATIONS (3)

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu g. \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/DNAP
Mean	8·54	6·33	0·75
S.E. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
S.E. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·6–17·4	0·3–1·8

Table 2. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in $\mu g. \times 10^{-7}$ per cell

		DNAP 28 obs. on 12 cases	RNAP	Ratio DNAP/RNAP 28 obs. on 13 cases
Group as a whole	Mean	12·6	10·9	0·87
	S.E.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
		12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
Group prior to therapy	Mean	12·57	13·38	1·06
	S.E.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
		17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
Group during the course of therapy	Mean	12·63	9·09	0·73
	S.E.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

Table 3. t *test of significance between means*

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	P	<0·001	<0·001	0·2–0·1
	Degrees of freedom	44 Highly significant	44 Highly significant	46 Not significant
Megaloblastic series before therapy compared with normal	P	0·01–0·001	<0·001	0·01–0·001
	Degrees of freedom	28 Highly significant	29 Highly significant	30 Highly significant
Megaloblastic series during therapy compared with normal	P	0·01–0·001	0·05–0·02	0·8–0·7
	Degrees of freedom	33 Highly significant	33 Significant	34 Not significant
Megaloblastic series before and during therapy compared	P	0·7–0·6	0·05–0·02	<0·001
	Degrees of freedom	27 Not significant	24 Significant	26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. Buffa, W. D. Lotspeich, R. A. Peters and R. W. Wakelin. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluorotricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

Table 1. *Rat heart aconitase*

Time (min.) ...	Citric acid (μmol.) 0	60
Additions:		
cis-Aconitate (5 μmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 μmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. *Pig heart isocitric dehydrogenase*

	$E_{340\,m\mu}$. (max. value)
DL-isocitrate only	0·076–0·065
Same + 'active' fraction	0·075
Same + *p*-chloromercuribenzoic acid $1·33 \times 10^{-3}$ M	0·004

Table 3. *Pig heart oxalosuccinic decarboxylase*

(CO_2 evolution from 10 μmol. oxalosuccinate in 30 min. at 13·5° C. Net values)

	CO_2 (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

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Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA Coding sequences Number of genes Active genes	~6 pg/cella 3% of genomic DNA 0.51.0 x 10^5 1.5 x 104
Total RNA rRNAs tRNAs, snRNAs, and low mol. wt. RNA mRNAs nuclear RNA	~10 50 pg/cellb 80 85% of total RNA 15 20% of total RNA 1 5% of total RNA ~14% of total RNA
Ratio of DNA:RNA in nucleus	~ 2:1
Number of mRNA moleculesc	0.2 1.0 x 10^6
Number of different mRNA species Low abundance mRNA (5 15 copies/cell) Intermediate abundance mRNA (200 400 copies/cell) High abundance mRNA (12,000 copies/cell)	1.0 3.4 x 10^4 11,000 different messages 500 different messages <10 different messages
Abundance of each message for: Low abundance mRNA (5 15 copies/cell) Intermediate abundance mRNA (200 400 copies/cell) High abundance mRNA (12,000 copies/cell)	<0.004% of total mRNA <0.1% of total mRNA 3% of total mRNA

a 30 – 60 µg/ml blood for human leukocytes.

b 1 – 5 µg/ml blood for human leukocytes.

c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells)	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

Table of Content

UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA ~ 3×10^6 nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (doiploid)	0.03 pg
E. coli	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	**Dimensions**	**Volume**
S. cerevisiae	5 µm	66 μm^3
S. pombe	2 x 7 µm	22 μm^3
Mammalian cell	10-20 µm	500-4,000 μm^3
E. coli	1 x 3 µm	2 μm^3
Mammalian mitochondrion	1 µm	0.5 μm^3
Mammalian nucleus	5-10 µm	66-500 μm^3
Plant chloroplast	1 x 4 µm	3 μm^3
Bacteriophage lambda	50 nm (head only)	6.6×10^{-5} μm^3
Ribosome	30 nm diameter	1.4×10^{-5} μm^3
Globular monomeric protein	5 nm diameter	6.6×10^{-8} μm^3

III. Some useful concentrations

Total cell protein concentration: Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

Abundant transcription factor	1 nM (100,000 copies/ nucleus)
Rare transcription factor	10 pM (1,000 copies/ nucleus)

Serum: 50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	**µg**
10,000 Da protein	1

100,000 Da protein	10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids ~/= 3.7 x 10^4 Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10-30 pg
Proportion of total RNA in nucelus	~ 14%
DNA:RNA in nucleus	~2:1
Human genome size (haploid)	3.3 x 10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5-1 x 10^5
Active genes	1.5 x 10^4
mRNA molecules	2 x 10^5 - 1 x 10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (µg)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*; cell, DNA, RNA, and protein content

	Leukocyes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O_2 & CO_2 transport
Cells per ml	4-7 x 10^6	3-4 x 10^8	5 x 10^9
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNa content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 106 per ml in cases of immunosuppression, to 40 x 10^6 during inflammation, to 500 x 10^6 during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

EVIDENCE APPENDIX

ITEM NO. 6
Second Supplemental Declaration
Of Andrew E. Lorincz
(filed in co-pending application SN10/179,589)
(Exhibit D)

EXHIBIT D

2nd Supplemental Declaration of Andrew E. Lorincz filed as Exhibit D of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia
Serial No.: 10/179,589
Filed: June 25, 2002
For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS

Group Art Unit: 1646
Examiner: Elizabeth Kemmerer

SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2. My Curriculum Vitae is attached was Exhibit A to my Declaration of November 8, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of June 5, 2006 provide additional information regarding my background and experience.

3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Second Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

> ...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Second Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Second Supplemental Declaration Exhibit C.

6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Second Supplemental Declaration Exhibit E. Note that in two of the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Second Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 4-19-07

Andrew E. Lorincz, M.D.

Andrew E. Lorincz, M.D.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

MARCH 7, 2007 OFFICE ACTION

Paragraph 11, pages 7-9

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6, and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo, ex vivo,* or *in vitro*.

EXHIBIT C

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1– 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEFG or BMP growth factors produced by VEFG or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEFG165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8–17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT D

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250 x 10^6 pg and 500 x 10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250 x 10^6 and 500 x 10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25 x 10^6 and 12.5 x10^6, respectively.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT E

PUBLICATIONS (3)

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu g. \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/DNAP
Mean	8·54	6·33	0·75
S.E. of obs.	2·89	3·03	0·326
Observed range	4·0–15·0	2·1–13·5	0·43–1·9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8·75	7·59	0·90
S.E. of obs.	3·05	3·72	0·30
Observed range	3·9–17·4	2·6–17·4	0·3–1·8

Table 2. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in $\mu g. \times 10^{-7}$ per cell

		DNAP	RNAP	Ratio DNAP/RNAP
Group as a whole		28 obs. on 12 cases		28 obs. on 13 cases
	Mean	12·6	10·9	0·87
	S.E.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
Group prior to therapy		12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
	Mean	12·57	13·38	1·06
	S.E.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
Group during the course of therapy		17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
	Mean	12·63	9·09	0·73
	S.E.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

Table 3. t *test of significance between means*

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	*P*	<0·001	<0·001	0·2–0·1
	Degrees of freedom	44 Highly significant	44 Highly significant	46 Not significant
Megaloblastic series before therapy compared with normal	*P*	0·01–0·001	<0·001	0·01–0·001
	Degrees of freedom	28 Highly significant	29 Highly significant	30 Highly significant
Megaloblastic series during therapy compared with normal	*P*	0·01–0·001	0·05–0·02	0·8–0·7
	Degrees of freedom	33 Highly significant	33 Significant	34 Not significant
Megaloblastic series before and during therapy compared	*P*	0·7–0·6	0·05–0·02	<0·001
	Degrees of freedom	27 Not significant	24 Significant	26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. Buffa, W. D. Lotspeich, R. A. Peters and R. W. Wakelin. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluorotricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

Table 1. *Rat heart aconitase*

Time (min.) ...	Citric acid (μmol.) 0	60
Additions:		
cis-Aconitate (5 μmol.)	0·21	3·90
cis-Aconitate + 'active' fraction	0·08	3·96
Citrate (5 μmol.)	4·90	4·34
Citrate + 'active' fraction	5·27	4·38

Table 2. *Pig heart isocitric dehydrogenase*

	$E_{340\ m\mu}$. (max. value)
DL-*isocitrate only*	0·076–0·065
Same + 'active' fraction	0·075
Same + *p*-chloromercuribenzoic acid $1·33 \times 10^{-5}$ M	0·004

Table 3. *Pig heart oxalosuccinic decarboxylase*

(CO_2 evolution from 10 μmol. oxalosuccinate in 30 min. at 13·5° C. Net values)

	CO_2 (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-*isocitrate (control)*	14

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Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	0.51.0 x 10^5
	1.5 x 104
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
Ratio of DNA:RNA in nucleus	~14% of total RNA
Number of mRNA moleculesc	~ 2:1
Number of different mRNA species	0.2 1.0 x 10^6
Low abundance mRNA (5 15 copies/cell)	1.0 3.4 x 10^4
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
Abundance of each message for:	<10 different messages
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

a 30 – 60 µg/ml blood for human leukocytes.

b 1 – 5 µg/ml blood for human leukocytes.

c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells)	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

Table of Content

UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA ~ 3×10^6 nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (doiploid)	0.03 pg
E. coli	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
S. cerevisiae	5 µm	66 μm^3
S. pombe	2 x 7 µm	22 μm^3
Mammalian cell	10-20 µm	500-4,000 μm^3
E. coli	1 x 3 µm	2 μm^3
Mammalian mitochondrion	1 µm	0.5 μm^3
Mammalian nucleus	5-10 µm	66-500 μm^3
Plant chloroplast	1 x 4 µm	3 μm^3
Bacteriophage lambda	50 nm (head only)	6.6×10^{-5} μm^3
Ribosome	30 nm diameter	1.4×10^{-5} μm^3
Globular monomeric protein	5 nm diameter	6.6×10^{-8} μm^3

III. Some useful concentrations

Total cell protein concentration: Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

- Abundant transcription factor: 1 nM (100,000 copies/ nucleus)
- Rare transcription factor: 10 pM (1,000 copies/ nucleus)

Serum: 50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	µg
10,000 Da protein	1

100,000 Da protein	10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids ~/= 3.7 x 10^4 Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10-30 pg
Proportion of total RNA in nucelus	~ 14%
DNA:RNA in nucleus	~2:1
Human genome size (haploid)	3.3 x 10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5-1 x 10^5
Active genes	1.5 x 10^4
mRNA molecules	2 x 10^5 - 1 x 10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (µg)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocyes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O_2 & CO_2 transport
Cells per ml	4-7 x 10^6	3-4 x 10^8	5 x 10^9
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNa content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 106 per ml in cases of immunosuppression, to 40 x 10^6 during inflammation, to 500 x 10^6 during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

EXHIBIT E

Third Supplemental Declaration of
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